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INTRASEXUAL SELECTION AND WARNING COLOR EVOLUTION IN AN APOSEMATIC POISON DART FROG

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INTRASEXUAL SELECTION AND WARNING COLOR EVOLUTION IN AN APOSEMATIC POISON DART FROG

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Dedication

In loving memory of my brother, Sean Crothers, and my grandfather, Randolph Hennes.

They shaped my life in ways that I could never possibly articulate,

and are dearly loved and dearly missed.

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INTRASEXUAL SELECTION AND WARNING COLOR EVOLUTION IN AN APOSEMATIC POISON DART FROG

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Flamboyant colors are widespread throughout the animal kingdom. While many of these traits arise through sexual selection, bright coloration can also evolve through natural selection. Many aposematic species, for example, use conspicuous warning coloration to communicate their noxiousness to predators. Recent research suggests these signals can also function in the context of mate choice. Studies of warning color evolution can therefore provide new insights into how the interplay of natural and sexual selection impact the trajectory of conspicuous signal evolution. For my dissertation, I investigated the potential for male-male competition to impact the warning color evolution of a species of poison frog. I focused my work on an exceptionally bright and toxic population of the strawberry poison frog (*Oophaga pumilio*) where males are brighter than females, a classic signature of sexual selection.

In Chapter 1, I used theoretical models of predator and frog visual systems to determine which can see the variation in bright warning coloration within this population. I found that birds, the presumed major predator, likely cannot see this variation, indicating that sexual selection can work under the radar of predators in this species. In Chapter 2, I tested the aggressive responses of males using a two-way choice paradigm that manipulated the perceived brightness of stimulus males. I found that males directed more of their behaviors to bright stimulus frogs, and brighter focal frogs more readily approached stimuli and directed more of their attention to the brighter rival. In Chapter 3, I tested the outcomes of dyadic interactions between males of varying brightness and observed male reactions to

simulated intruders in their territories. I found that brighter males initiated aggressive interactions with rivals more readily, and brightness asymmetries between males settled interactions in a way that is consistent with classic hypotheses about male sexual signals. In Chapter 4 I sought to describe physiological correlates of male warning color brightness. While male brightness did not co-vary with classic measures of body condition (circulating testosterone and skin carotenoids), it did correlate with toxins sequestered from the diet and thus appears to be a reliable signal of toxicity in this population.

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INTRODUCTION

Humans have admired and puzzled over conspicuous animal signals for millennia (Darwin 1871; Ovid 2008). While sexual selection often drives the evolution of conspicuous traits (Andersson 1994), signals can also evolve through natural selection to communicate unprofitability to potential predators (Wallace 1867; Mallet and Joron 1999; Ruxton et al. 2004; Mappes et al. 2005). These "aposematic" signals are widespread throughout the animal kingdom, and represent one of the most accessible traits to evaluate the interaction of natural and sexual selection on signal evolution. Because aposematic species are highly conspicuous and can simultaneously signal to both predators and conspecifics with the same trait (Jiggins et al. 2001; Maan and Cummings 2008; Nokelainen et al. 2011), investigating how these viewers select on aposematic traits can help clarify the underlying principles governing the evolution of signals.

Importantly, studies of signal evolution in aposematic species can be seen as complementary to studies in non-aposematic organisms. Investigations of trait evolution in non-aposematic species typically focus on how natural selection may limit the evolution of a sexually selected signal (e.g., Endler 1983). In contrast, investigations of trait evolution in aposematic species can focus on how sexual selection impacts signals that have arisen through natural selection (e.g., Jiggins et al. 2001), effectively flipping on its head the question of how conspicuous trait evolution happens. Such empirical investigations, working in tandem, can elucidate how Charles Darwin's two processes of selection (natural and sexual) impact the diversification of signals in general, and even drive speciation (Maan and Seehausen 2011).

The Study Species

The strawberry poison frog, *Oophaga [Dendrobates] pumilio,* is one of the most phenotypically variable of the Dendrobatidae family of poison frogs. *O. pumilio* is monomorphic in coloration across most of its geographic range spanning Nicaragua to Panama, with a reddish body and blue legs. However, in the Bocas del Toro archipelago,

located in the Caribbean on the western end of Panama, the species exhibits dramatic variation in hue and brightness. Across island populations and throughout the mainland of this region, the species exhibits an estimated 15-30 unique color patterns (Daly and Myers 1967; Siddiqi et al. 2004).

At first blush, the geographical isolation of the populations of Bocas del Toro seems to implicate neutral vicariance mechanisms in the diversification of *O. pumilio*'s aposematic coloration. However, the islands of the archipelago formed recently, as sea levels rose during the Holocene. It has thus been estimated that these *O. pumilio* populations became isolated over the course of the last 9,000 years (Anderson and Handley 2001). A number of investigations have found no evidence that genetic drift accounts for the divergence of color patterns in the species, and strong support for the role of selection in causing this diversification (Summers et al. 1997; Hagemann and Pröhl 2007; Rudh et al. 2007; Brown et al. 2010; Wang and Summers 2010; though see Gehara et al. 2013). However, while genetic drift has largely been ruled out as a major source of this variation, the specific selective forces that produced it remain unclear. The recent color diversification in this species provides a unique natural experiment, allowing investigators to assess the relative strengths and roles of natural and sexual selection in driving aposematic signal diversification.

Evidence for Natural Selection

The potential role for natural selection in the divergence/maintenance of phenotypic variation across *O. pumilio* populations is relatively unknown. It has been suggested that predators were not a strong diversifying force in the species' color evolution, as two syntopic species of poison frog are not polytypic (Summers et al. 1997) and there is no evidence that *O. pumilio* is part of a Müllerian mimicry complex (Siddiqi et al. 2004). Traditionally, natural selection is predicted to favor convergence across aposematic systems (Müller 1879); color pattern convergence reduces the signal combinations for predators to learn and thus lowers the number of sacrificial individuals necessary to 'train' predators. However, recent research suggests that several processes may relax stabilizing selection on aposematic signal design. Ecological factors such as the signaling environment, variation in predator communities or dietary alkaloid availabilities may contribute to variation in aposematic signal form (Mallet

and Joron 1999; Speed 1999; Endler and Mappes 2004; Ruxton et al. 2004; Mappes et al. 2005; Speed and Ruxton 2007).

For example, ecological constraints may contribute to the divergence of aposematic signals by imparting selection for crypsis. As populations inhabit new environments that are limited in chemical defense resources, aposematic signal diversification may be driven away from conspicuousness and towards cryptic forms if predators impose selection for honest aposematic signaling (Sherratt 2002; Blount et al. 2009; Cummings and Crothers 2013). Poison frogs derive their toxicity from alkaloids sequestered from their diet of arthropods (Daly et al. 1994; Saporito et al. 2004; Saporito et al. 2007a; Saporito et al. 2012), hence variation between sites in arthropod communities may lead to changes in toxicity levels between populations (Saporito et al. 2006; Saporito et al. 2007b). Recent evidence indicates that brightness correlates strongly with toxicity across phenotypically distinct *O. pumilio* archipelago populations, and that avian visual systems can detect this relationship better than other viewers (snakes, crabs, conspecifics: Maan and Cummings 2012). Furthermore, recent empirical evidence suggests that avian predators in Bocas del Toro respond to signal differences in *O. pumilio* morphs. Using clay model replicas, Hegna et al. (2012) showed that birds attack a less toxic/less conspicuous morph more often than a more toxic/more conspicuous morph. Hence, as populations of *O. pumilio* became isolated on islands with potentially different predator/arthropod communities, the selection pressure along the crypsis-conspicuous continuum appears to have varied.

For predators that are able to learn to avoid aposematic prey, their learning biases may favor enhanced aposematic signal conspicuousness. Several studies have shown that increases in conspicuousness of aposematic signals can lead to more rapid predator learning (Gittleman and Harvey 1980; Sillén-Tullberg 1985; Endler and Mappes 2004; Darst et al. 2006; Gamberale-Stille et al. 2009). Furthermore, brightness contrast alone has been shown to serve as an effective warning signal, and can be important in the initial stages of aposematic learning (Prudic et al. 2007; Sandre et al. 2010). Hence, natural selection may act additively or synergistically with sexual selection (described below) by favoring brighter, more conspicuous phenotypes in some *O. pumilio* populations (Cummings and Crothers 2013).

Natural selection may also be largely permissive of signal diversification promoted by other factors (e.g., sexual selection) due to psychological or sensory constraints of predators. Variations in aposematic signals may not suffer purifying selection due to predators' neophobia (Marples et al. 1998) or because predators generalize their learned avoidance to novel forms (Pavlov 1927; Darst and Cummings 2006; Exnerova et al. 2006). Experimental studies with bird predators and other poison frog species (*Epipedobates sp.*) have shown that predators respond to novel forms of aposematic prey in a toxicity-dependent manner. Specifically, predators familiar with more toxic prey species avoid novel prey forms, whereas predators familiar with less toxic prey do not avoid novel morphs (Darst and Cummings 2006). Hence, new aposematic variants may be protected through a process of relaxed purifying selection due to stimulus generalization, or perhaps simply because predator sensory systems are unable to detect that variation.

Evidence for Sexual Selection

The unique life history of *O. pumilio* provides a strong indication that sexual selection is at play in the species, and sexual selection has been favored as the major mechanism promoting the species' color diversification (reviewed in Cummings and Crothers 2013 and Gehara et al. 2013). Males of the species are highly territorial, exhibiting territory site fidelity (McVey et al. 1981), and guarding areas that contain sites for foraging, tadpole rearing, and locations to perch and call to females (Donnelly 1989; Pröhl 1997). Males are known to vigorously defend their sites against conspecific males through vocalizations and close-range aggressive encounters (Bunnell 1973; Forester et al. 1993; Baugh and Forester 1994; Gardner and Graves 2005; Pröhl 2005). Like many dendrobatids (Weygoldt 1980), *O. pumilio* provides extended parental care to its offspring, and courtship is extensive (Limerick 1980). During mating, females lay eggs in males' territories, which males guard and keep hydrated for approximately one week (Tazzyman and Iwasa 2010). Beyond this, females provide a disproportionate amount of parental care, rearing tadpoles after they hatch by carrying them to water-filled leaf axils and feeding them with unfertilized eggs for approximately 7 weeks until the tadpoles metamorphose (Weygoldt 1980; Pröhl and Hödl 1999). Furthermore, there is evidence for very high reproductive skew in the species, with great variance in male mating

success (Pröhl and Hödl 1999). Together, these factors likely result in strong intrasexual competition for mating success (Trivers 1972).

Females are known to select for enhanced male signals (louder, brighter, bigger) in a variety of taxa (Ryan and Keddy-Hector 1992; Andersson 1994). Furthermore, sexual selection on an ecologically important trait such as warning coloration is also expected to result in sexual dimorphism in the trait (Lande and Arnold 1985). Coincident with theory, many of the *O. pumilio* morphs inhabiting the islands of the Bocas del Toro region are brighter than the presumed ancestral morph found on the mainland, and sexual dimorphism in brightness has been identified in at least one population, suggesting a role for directional selection on brightness in the populations of the archipelago (Maan and Cummings 2009). Summers and colleagues hypothesized that pre-existing mate preferences might drive rapid divergence in color across this group (Summers et al. 1997; Siddiqi et al. 2004) and Tazzyman and Iwasa (2010) theorized that this could be achieved through a process of "coupled drift," in which selection causes coloration to follow the trajectory of female preference, which largely evolves via drift. Alternatively, research examining a simple preference for brighter males across populations of the archipelago suggests that directional selection for brighter phenotypes may contribute color diversification across populations, because selection on brightness often results in concomitant changes in hue (Maan and Cummings 2009; Crothers and Cummings 2013).

A previously unexplored selective agent in aposematic communication is male conspecifics, which may attend to these signals during male-male competitive interactions, especially in highly territorial aposematic species like *O. pumilio*. This possibility is especially probable given the well-documented roles that conspicuous, non-aposematic signals play in territorial behaviors (Andersson 1994; Berglund et al. 1996), and accumulating evidence that warning signal expression is linked to metabolic and physiological phenotype (Crothers et al. 2011; Santos and Cannatella 2011; Pegram et al. 2013), potentially providing conspecifics with information regarding competitive ability and vigor. Male competition is believed to have produced polymorphisms in many non-aposematic systems (e.g., Zamudio and Sinervo 2000; Dijkstra et al. 2005; Pryke and Griffith 2006), and can lead to speciation (Seehausen and Schluter 2004). Studies of aposematic signal evolution in species with male competition

are thus likely to be particularly rewarding for studying the interplay of natural and sexual selection on the trajectory of conspicuous signal evolution.

Here, I investigate potential behavioral, physiological and ecological underpinnings of aposematic signal variation in this model species for signal variation. I focus my research on an exceptionally bright and sexually dimorphic population in order to assay which viewers may be driving the enhanced aposematic conspicuousness found within this population. To investigate these questions, my dissertation research integrates behavioral tests (to determine how sexual selection may impact signal evolution), chemical ecology (to determine if there is a correlation between brightness and toxicity/skin pigments within a population), sensory ecology (to examine warning signal brightness discriminability to different viewers), and physiological measures (to determine whether circulating testosterone co-varies with brightness and/or behavior). My research represents the first investigation of the role that male-male competition can play in aposematic signal evolution, and thus the first integrating the many potential viewers/selective agents of this trait. These studies provide insights into the selective forces maintaining and contributing to warning color diversity, and clarify how evolutionary feedbacks between sexual selection and natural selection can drive the evolution of conspicuous signals in animals.

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CHAPTER 1:

Sexual selection may work under the radar of natural selection in populations of a polytypic poison frog

ABSTRACT

Though theory predicts consistency of warning signals in aposematic species to facilitate predator learning, variation in these signals often occurs in nature. The strawberry poison frog, *Oophaga [Dendrobates] pumilio*, is an exceptionally polytypic (populations are phenotypically distinct) aposematic frog exhibiting variation in warning color and brightness. In the Solarte population, males and females both respond differentially to male brightness variation. Here, we demonstrate through spectrophotometry and visual modeling that aposematic brightness variation within this population is likely visible to two putative predators (crabs, snakes) and conspecifics, but not to the presumed major predator (birds). This study thus suggests that signal brightness within *O. pumilio* populations can be shaped by sexual selection, with limited opportunity for natural selection to influence this trait due to predator sensory constraints. As signal brightness changes can ultimately lead to changes in hue, our findings at the within-population level can provide insights into understanding this polytypism at across-population scales.

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1.1 INTRODUCTION

Conspicuous traits typically evolve through an antagonistic interplay between sexual selection and predation (Darwin 1887; Endler 1992). Yet in aposematic species, which use "warning" signals to deter predators (Ruxton et al. 2004), the interaction of these forces on conspicuousness is not as clear. Considerable intra- and inter-population variation in warning coloration has been observed across a diversity of taxa (e.g., Bezzerides et al. 2007; Speed et al. 2010; reviewed in Ruxton et al. 2004). Recent work suggests that aposematic traits can be co-opted as sexual signals (Jiggins et al. 2001) and evolve via sexual selection (Maan and Cummings 2009). Since these traits often advertise to multiple predators featuring diverse sensory systems (Endler and Mappes 2004), variation in aposematic signals driven by sexual selection is predicted to be opposed by predators (Müller 1879). However, the interaction between these two selective forces on such signals is relatively unknown, and the perceptibility of aposematic signal variation to predators remains underinvestigated (Stevens 2007; Lindstedt et al. 2011; Maan and Cummings 2012).

The aposematic strawberry poison frog, *Oophaga [Dendrobates] pumilio*, exhibits extreme warning color variation in the Bocas del Toro archipelago of Panama, with ~15 distinct phenotypes represented across island and mainland populations ("polytypism"; Daly and Myers 1967; Siddiqi et al. 2004). Assortative mating of color morphs within populations and directional sexual selection on male coloration and brightness across populations have both been implicated in the evolution of this variation (Summers et al. 1999; Reynolds and Fitzpatrick 2007; Rudh et al. 2007; Maan and Cummings 2008, 2009). One of the best-studied populations (Solarte) contains orange-red frogs representing one of the brightest morphs in the archipelago (Fig 1.1a; 2nd brightest of 10 populations, Maan and Cummings 2012). While both Solarte males and females are exceptionally bright, this population is also sexually dimorphic in terms of aposematic brightness and both sexes exhibit differential behavioral responses to male brightness variation found within the population (Maan and Cummings 2009; Crothers et al. 2011). Taken together, these results suggest that this phenotypic feature is under the influence of sexual selection. Here, we examine whether this

aposematic brightness variation, likely generated by sexual selection, is detectable to the putative predators of *O. pumilio*.

Purifying selection against rare aposematic phenotypes has been observed in polymorphic insects (Kapan 2001; Borer et al. 2010) and other polytypic poison frog species (Noonan and Comeault 2009; Chouteau and Angers 2011; Comeault and Noonan 2011), suggesting that such forces could also be at play in *O. pumilio*. Natural selection by predators may therefore interact with sexual selection in this species, effectively limiting any deviations from the average aposematic phenotype within a given population. Though the main predators of *O. pumilio* in the Bocas del Toro region are still unknown, clay frog predation experiments have implicated birds as the major predator of several poison frog species (Noonan and Comeault 2009; Chouteau and Angers 2011; Comeault and Noonan 2011) as well as *O. pumilio* in other parts of the species' geographic range (Saporito et al. 2007; Hegna et al. 2011). While birds are capable of detecting differences between the different *O. pumilio* morphs (Maan and Cummings 2012; M. Cummings, *unpublished data*), no studies have yet investigated whether within-population variation in brightness is also detectable to predators. Such investigations can elucidate the relative roles played by natural selection and sexual selection in the phenotypic divergence of this species. Here, we use taxon-specific visual modeling analyses of the conspecific and three predator visual systems, using spectrophotometric measurements of Solarte *O. pumilio* males and six perch backgrounds, to investigate the perceptibility of intra-population aposematic brightness variation. This study aims to *(1)* determine whether potential predators are likely to perceive brightness variation in male *O. pumilio* of the Solarte population, and *(2)* determine whether variation in perch site backgrounds affects the discriminability of this variation in male conspicuousness.

1.2 METHODS

Collection and Spectral Measurements

Solarte *O. pumilio* males (N=128) were captured in July-August 2009 and measured in a temperature-controlled room at the Smithsonian Tropical Research Institute, in Bocas del Toro, Panama, within 24 hours of capture. Spectral reflectance measurements were taken at

the head and dorsum (two measurements per region) using an EPP200C UV-VIS spectrometer, SL-4 Xenon lamp, and R400-7 reflectance probe, at a 3mm distance for frogs, and 2mm distance for substrates (StellarNet Inc., Tampa, FL). Spectralon white standard measurements were taken between frogs to account for lamp drift.

Perceptually Unbiased Measures

Dorsal measurements (N=4) were averaged for each frog. We first calculated inherent measures of brightness (log of total reflectance flux): $[\log(\sum_{i=300nm}^{700nm} R(\lambda)_i)]$ and color (long-wave chroma) to evaluate brightness and coloration using spectrophotometry. Long-wave chroma assesses the proportion of the reflectance flux in the long-wave band (600-700nm): $\left[\frac{\sum_{i=600nm}^{700nm} R(\lambda)_i}{\sum_{i=300nm}^{700nm} R(\lambda)_i}\right]$

Lastly, we calculated the inherent radiance contrast of a frog against its background

$[(Q_{frog} - Q_{background})/(Q_{frog} + Q_{background})]$ where $Q = \int_{\lambda=300}^{700} I(\lambda)R(\lambda)d\lambda$ and $I(\lambda)$ is the habitat irradiance and $R(\lambda)$ is the reflectance.

Taxon-Specific Measures

To assess the perceptual consequences of brightness variation in a taxon-specific manner, we estimated the contrast of frogs against different backgrounds using receptor-based visual models. Visual models provide an approximation of an animal's perception of visual information, have predicted behavioral sensitivities in a variety of taxa (Vorobyev and Osorio 1998; Vorobyev et al. 2001; Goldsmith and Butler 2003), and can provide reliable estimates of predation risk (Stuart-Fox et al. 2003; Husak et al. 2006; Stobbe and Schaefer 2008).

Frog predators come from widely different taxa (e.g., Silverstone 1975; Myers and Daly 1976). Accounts of attacks on *O. pumilio*, though rare, implicate forest crabs, birds, spiders, and snakes as potential predators (M. Cummings, *unpublished data*). Our predator visual models include previously described passerine, crab, and diurnal snake models (Cummings et al. 2008; Maan and Cummings 2012), as well as a conspecific visual model

(based on Siddiqi et al. 2004; Maan and Cummings 2009, 2012; Crothers et al. 2011). Our visual modeling methods are based on receptor noise-limited models originally developed by Vorobyev and Osorio (1998). Each model involves steps and equations described previously (Cummings et al. 2008; Maan and Cummings 2009, 2012), incorporating frog and background reflectance, ambient light, and receiver visual sensitivities into conspicuousness calculations, and assuming that visual detection ability is limited by photoreceptor noise (data deposited in the Dryad repository: doi:10.5061/dryad.p5g5j). In brief, the models use the following steps:

Photoreceptor quantum catch, Q_c, for target (frog) or background (substrate) radiance is calculated as the integrated product of habitat irradiance, $I(\lambda)$, target or background reflectance ($R(\lambda)$), and photoreceptor cone absorbance ($A(\lambda)$) for each cone class, c:

$$Q_C = \int_{\lambda=300}^{700} I(\lambda)R(\lambda)A_c(\lambda)d\lambda$$

integrated over 1 nm intervals from 300 to 700nm. Quantum catch is adjusted for the adapting light environment using von Kries transformations, such that $q_c = k_c Q_c$ and

$$k_c = 1/(\int_{\lambda=300}^{700} I_b(\lambda)A_c(\lambda)d\lambda)$$

where $I_b(\lambda)$ is the adapting visual background (=habitat irradiance). Photoreceptor signal is proportional to the logarithm of these adjusted quantum catches such that contrast between target and background is

$$\Delta f_c = \ln[q_c(target)] \,/\, [q_c(background)]$$

where background was one of six common substrates in male territories. Substrates used in the analyses were the most common daytime signaling backgrounds used by calling males in this population (see also Pröhl and Ostrowski 2011). We quantified perch site substrate use by finding 82 calling males across June-August of 2011. Calling males were located at their perch sites, and the substrate background upon which the male was standing was noted. Substrate samples were then collected for subsequent spectral reflectance measurement. The substrates were then organized into six classes that encapsulate the diversity of spectral

characteristics of calling backgrounds in this population: moss (13 males), leaf litter (16), green leaves (11), dry fallen palm leaves (24), soil (5), and tree trunks/branches (13) (see Fig 1.2 for spectra). All visual model analyses were evaluated using a habitat irradiance representing the median quantum flux measurement collected across 38 Solarte male territories on an overcast day in August 2009.

Target detection is assumed to be subject to photoreceptor noise (ω), a function of the weber fraction for each cone class (ν) and the relative number of receptor types in the retina (η), where $\omega = \nu/\eta$. Cone proportions and weber fractions used for these models have been described previously (Maan and Cummings 2009; Maan and Cummings 2012).

Color and brightness appear to be processed independently in invertebrates and in vertebrates (Fleishman and Persons 2001; Endler and Mielke 2005; Osorio and Vorobyev 2005; Lind and Kelber 2011), thus we calculated two separate contrast measures: brightness contrast and chromatic contrast. Brightness contrast (ΔL), the ability to discriminate target from background in the luminance channel, is governed by the long-wavelength sensitive (LWS) cone class in many terrestrial organisms (Maier and Bowmaker 1993), and by the double cones in birds (Endler and Mielke 2005). Signal to noise estimates in the luminance channel were therefore evaluated as $\Delta L = \left| \Delta f_{LWS} / \omega_{LWS} \right|$ for frog, snake, and crab models, and $\Delta L = \left| \Delta f_{double} / \omega_{double} \right|$ for the bird using the double cone spectral absorbance measures of the Starling (kindly provided by N. Hart; Hart et al. 1998). This is a departure from our laboratory's previous modeling investigations wherein the LWS cones were used for avian brightness contrast estimates (Maan and Cummings 2009, 2012). Chromatic contrast estimates (ΔS) were evaluated according to the type of visual system, using the equations below:

Dichromat (crab): $\Delta S = \sqrt{(\Delta f_L - \Delta f_S)^2/(\omega_S^2 + \omega_L^2)}$

Trichromat (snake, frog): $\Delta S = \sqrt{\frac{(\omega_S^2(\Delta f_L - \Delta f_M)^2 + \omega_M^2(\Delta f_L - \Delta f_S)^2 + \omega_L^2(\Delta f_S - \Delta f_M)^2)}{(\omega_S\omega_M)^2 + (\omega_S\omega_L)^2 + (\omega_M\omega_L)^2}}$

Tetrachromat (bird):

$\Delta S =$

$$\sqrt{\frac{(\omega_U\omega_S)^2(\Delta f_L-\Delta f_M)^2+(\omega_U\omega_M)^2(\Delta f_L-\Delta f_S)^2+(\omega_U\omega_L)^2(\Delta f_M-\Delta f_S)^2+(\omega_S\omega_M)^2(\Delta f_L-\Delta f_U)^2+(\omega_S\omega_L)^2(\Delta f_M-\Delta f_U)^2+(\omega_M\omega_L)^2(\Delta f_S-\Delta f_U)^2)}{(\omega_U\omega_S\omega_M)^2+(\omega_U\omega_S\omega_L)^2+(\omega_U\omega_M\omega_L)^2+(\omega_S\omega_M\omega_L)^2}}$$

In addition to calculating brightness (ΔL) and chromatic contrasts (ΔS), we also calculated an "overall conspicuousness" measure, represented as the combined contrast of both ΔL and ΔS and evaluated as the Euclidean distance from the origin in perceptual space, with ΔL on the x-axis and ΔS on the y-axis (as in Fig 1.1). Estimates of <1 are considered indistinguishable (less than the signal detection threshold).

Data Analysis

We first assessed signaling backgrounds of calling males of this population using a Chi-square goodness-of-fit test, testing the hypothesis that frogs were distributed equally across substrate categories. We then used visual modeling estimates to assess the ability of viewers to distinguish differences between males, using two approaches: *(a)* a comparison of all possible male pairs across the population, and *(b)* comparison of dull vs. bright male classes. For the first approach we created distance matrixes of pairwise Euclidian distances between all males in the dataset against each of the 6 backgrounds. Wilcoxon signed rank tests were then performed to see if these distributions of Euclidian distances exceeded 1, allowing us to assess whether population-wide variation in brightness was detectable to these different viewers. We then classified males by their inherent brightness into above ("Bright"; N=64) or below ("Dull"; N=64) population mean log total reflectance flux, and evaluated these categories with each taxon-specific visual model to determine whether the variation in male brightness between these two classes of males was distinguishable to different viewers. ΔS and ΔL estimates were thus calculated for all males with each of the substrate backgrounds and taxon-specific visual models. The resulting estimates were then compared between the bright and dull male categories using Wilcoxon rank sum tests to assess if the male brightness classes differed by more than the signal detection threshold (null mu <1). Finally, to assess how the substrate background affected each individual's contrast estimates, we performed paired Wilcoxon signed rank tests (null mu <1) for each individual across the

six substrate backgrounds for each of the taxon-specific visual models. All analyses were performed in R (R Development Core Team 2012). P-values were corrected for multiple comparisons using the Bonferroni-Holm correction.

1.3 RESULTS

Perch Site Substrates

Males were not equally distributed among the substrate categories (Chi square test; χ^2=14.29, df=5, P=0.014). Post hoc investigation indicated that males were found significantly more often on a palm background, and less often on a soil background than expected by chance (standardized residuals >|2|).

Perceptually Unbiased Measures of Contrast

Brightness and long-wave chroma (~saturation of redness) exhibited a negative relationship (Fig 1.1a; t=-9.158, P<<0.001, R^2: 0.3996). As expected, inherent contrast estimates between the bright and dull male categories were non-overlapping and differed significantly against all substrate backgrounds (Table 1.1; Wilcoxon rank sum tests, all P << 0.001).

Taxon-Specific Measures

Pairwise Euclidian distance estimates for all possible male pairings, an assessment of the distinguishability of spectral variation between males, exceeded the signal detection threshold for all visual systems but the bird (gray values in Table 1.2; Wilcoxon signed rank tests, all P << 0.001 for crab, snake, conspecific; P=1 for bird), indicating that much of the variation between males is likely perceptible to these visual systems but not to avian predators. Analysis of the two inherent male brightness classes (bright and dull) revealed that brightness contrast (ΔL) estimates between males of these categories differed by more than the signal detection threshold against all substrate backgrounds for the crab, snake, and conspecific visual models, but not for the bird (Wilcoxon rank sum tests, all P << 0.001 for crab, snake, conspecific; P=1 for bird; Table 1.1). Furthermore, only a portion of the bright

class, and none of the dull, was detectable to the avian viewer in the ΔL channel against the background upon which males were most commonly found (dry palm; Fig 1.1b; Fig 1.3; Table 1.1). None of the chromatic (ΔS) comparisons between the bright and dull classes differed by more than the signal detection threshold for any of the visual models (Fig 1.1b; Table 1.1).

Overall conspicuousness of the bright and dull classes differed by more than the signal detection threshold for the snake, crab, and conspecific visual models against several substrate backgrounds (Wilcoxon rank sum tests: frog: moss, tree, soil (all Ps < 0.001); snake: all substrates (all Ps < 0.01); crab: moss, tree, soil (all Ps < 0.001)), but never for the bird visual model (all Ps = 1). Overall conspicuousness measures therefore match our findings using solely ΔL or ΔS in almost all analyses (Table 1.2; Fig 1.4). Thus, our modeling indicates that even when evaluating overall conspicuousness, a more holistic estimate of perceptual ability, birds are unable to distinguish male brightness variation, while the other predator and conspecific visual systems can under several viewing conditions (Table 1.2). We found that for the crab and conspecific visual systems, all frogs exceeded the signal detection threshold in terms of overall conspicuousness. For the snake and bird visual systems, a small fraction of frogs did not exceed the overall conspicuousness signal detection threshold against a background of green leaves (5% for snake) and palm leaves (0.8% for snake and bird; Fig 1.1b; Table 1.2).

Assessments of Background Effects on Individual Males' Conspicuousness

Our paired Wilcoxon tests indicated that individual frogs' contrast estimates varied perceptibly (>signal detection threshold) against several of the different substrate backgrounds (Fig 1.4).

1.4 DISCUSSION

Our study indicates that *(1)* the extensive inter-male variation in brightness in the *O.*

pumilio Solarte population is likely detectable to conspecifics and some predators (snakes, crabs), but not to the presumed major predator (birds), and *(2)* these results are robust to the naturally occurring backgrounds upon which males are commonly found (Fig 1.1; Table 1.1). While these findings are based on theoretical visual modeling, studies comparing receptor-noise limited models with behaviorally measured sensitivities have demonstrated these models' usefulness in birds (e.g., Goldsmith and Butler 2003) and other taxa (Vorobyev et al. 2001). Our frog model results also corroborate behavioral responses of conspecifics to brightness variation in this population ("bright" vs. "dull" males; Maan and Cummings 2009; Crothers et al. 2011). Hence, these results likely provide realistic estimates for actual perceptual differences in the wild.

There is mounting evidence that variation in aposematic coloration is common (Fig 1.1; Bezzerides et al. 2007; Speed et al. 2010), and that both color (reviewed in Ruxton et al. 2004) and brightness (Prudic et al. 2007) are important signal components that predators attend to. In *O. pumilio*, male brightness is a salient cue during male territorial interactions (Crothers et al. 2011) and may be undergoing directional sexual selection in at least some populations of this species (Maan and Cummings 2009). Though sexual selection has evidently impacted the evolution of coloration in *O. pumilio*, the species' conspicuous phenotype also functions as an aposematic signal (Saporito et al. 2007). Phenotypic variation has not been observed in other syntopic poison frog species (Summers et al. 1997). However, the variation in dorsal brightness across *O. pumilio* populations in Bocas del Toro appears both detectable and informative (in terms of toxicity level) to potential predators, particularly birds (Maan and Cummings 2012), suggesting a potential for predators to influence this color variation across the archipelago. Yet interestingly, our study suggests that these same predators (birds) are unlikely to detect the variation in signal brightness within one of the brightest populations.

Birds are considered an important predator of poison frogs (Noonan and Comeault 2009; Chouteau and Angers 2011; Comeault and Noonan 2011), including *O. pumilio* (Saporito et al. 2007; Hegna et al. 2011). Therefore, the information provided by Solarte's brightness variation, though accessible to conspecifics, appears to be indiscriminable to its presumed major predator (Fig 1.1b; Fig 1.3; Table 1.1). However, despite this sensory

constraint, it is possible for avian predators to exert selection on brightness variation in this population indirectly. Brighter Solarte males approach rival males faster than duller males (Crothers et al. 2011). These behavioral correlates of brightness may allow avian predators to exert selective pressure on brightness by differentially preying upon more active males.

To understand the mechanisms driving the evolution of warning signals, it is necessary to analyze warning phenotypes not only within the framework of predator perception but also against common signaling backgrounds. We found that Solarte males' brightness variation was always discriminable to the conspecific, snake, and crab visual systems, but never to the bird visual system, against all of the signaling backgrounds that we identified (ΔL; Table 1.1). Brightness and chromatic components of a visual scene are often used in different ways. Brightness information is typically used to detect small objects, track movement and resolve pattern details, while chromatic information is used to identify the spectral features of materials and discriminate large targets (Osorio et al. 1999; Jones and Osorio 2004; Endler and Mielke 2005). It is therefore assumed that achromatic (brightness) vision dominates at large distances, while chromatic information is accessible when an animal is closer to its target (Campenhausen and Kirschfeld 1998; Osorio et al. 1999; Defrize et al. 2010; though see Schaefer et al. 2006, Stobbe et al. 2009; Lind and Kelber 2011). This implies that at a distance many frogs may be undetectable (especially on palms, their most common signaling background), and that signal variation between males in this population may be particularly indistinguishable to birds (Table 1.1; Fig 1.3).

Of the six substrates on which our sample of *O. pumilio* males were found calling, significantly more males were found calling on palm leaves than expected by chance. This substrate renders males less conspicuous (less detectable) than other substrates to the conspecific and predator visual systems examined here (Table 1.1; Fig 1.3; Fig 1.4), though our methods did not allow us to distinguish whether this substrate is more plentiful in the Solarte environment, if frogs are choosing particular signaling backgrounds, or other scenarios.

Our results also indicate that the substrate background can have profound effects on not only an individual's perceived conspicuousness, but also on their conspicuousness

relative to other individuals in the population. For instance, exceptionally dull males (lower total reflectance) are relatively more conspicuous than exceptionally bright males (higher total reflectance) on green leafy backgrounds, while having the reverse relationship on several other backgrounds (Fig 1.4). Because the relative conspicuousness rankings of individuals within this population can depend strongly on signaling background, substrate choice is likely to have fitness consequences (Bateson and Healy 2005). Additionally, given that brightness and chromatic information are likely used in different ways, our results suggest that a large percentage of duller than average frogs are cryptic at a distance when signaling on a background of green leaves, palm leaves, or leaf litter (ΔL values below 1 for all visual models; see Fig 1.1b; Fig 1.3; Table 1.1). Solarte males were most often found on palm leaves, a substrate on which a proportion of the population are expected to be indiscriminable, for all visual systems, in terms of brightness ($\Delta L < 1$), but discriminable in terms of color and overall conspicuousness (Table 1.1; Table 1.2). Several studies indicate that aposematic prey can be cryptic at a distance and conspicuous at close range (Papageorgis 1975; Tullberg et al. 2005; Gamberale-Stille et al. 2009; Defrize et al. 2010), a strategy that may be used in this population.

The mechanisms maintaining Solarte brightness variation remain unclear, yet the current data favor sexual selection as a major contributor. Research into the physiological correlates of dorsal brightness in this population suggests that brightness may be a condition-dependent trait. Solarte male brightness does not correlate with body mass, length, or a traditional metric of condition (length-mass residuals), however, it does correlate with body temperature, an important trait in ectotherms (Crothers et al. 2011). Furthermore, the coloration and brightness of red/orange *O. pumilio* morphs can change in captivity over long time periods, indicating that at least some component of coloration may be dietarily based (L. Crothers, *unpublished data*; Summers et al. 2003; J. Yeager and C. Richards-Zawacki, *unpublished data*). If dorsal brightness is condition-dependent in this population, then we should not be surprised to see that both males and females exhibit variation in this trait (see Maan and Cummings 2009). Furthermore, the sexual dimorphism we observe in this trait is also expected in this species, where males are under greater sexual selection pressures than

females through the combined effect of females being choosier (due to greater parental investment) and males experiencing strong intrasexual selection (Pröhl and Hödl 1999).

Finally, the results of our study, though limited to a population exhibiting a single color pattern, provide insights into the selective mechanisms of the species' color diversification. Though the relative roles of male- and female-mediated sexual selection are under active investigation, if sexual selection on *O. pumilio*'s aposematic phenotype continues in a unidirectional fashion to enhance male brightness, chromatic aspects of the signal are also likely to be impacted. Maan and Cummings (2009) hypothesized that interdependence between brightness and hue in the aposematic coloration of *O. pumilio*, coupled with sexual selection on small, isolated populations, may account for the change in hue observed across island populations of Bocas del Toro. Our measurements here show that changes in inherent brightness result in a concomitant change in chromatic properties within a *single* population. We found a negative relationship between long-wave chroma (~redness) and brightness (Fig 1a) in the Solarte population, and a complementary trade-off between chromatic contrast and brightness contrast for the taxon-specific viewers (Fig 1.1b). This coupling of chroma and brightness suggests that the previously documented sexual selection on brightness could play a key role in the diversification of hues in isolated populations of this species.

Our study suggests that natural selection and sexual selection may predominantly be impacting aposematic coloration at different scales in *O. pumilio*. The inability of the species' major predator (birds) to detect the extensive brightness variation found within this population hints at a permissiveness of predators towards ongoing signal evolution promoted by conspecifics. Thus, variation in signal brightness may be shaped by sexual selection working below the radar of natural selection at the within-population ("micro") scale. However, this trait is evidently influenced by natural selection at a larger ("macro") scale across populations, where there is a strong relationship between morph toxicity and dorsal brightness across *O. pumilio* morphs, and this relationship appears to be more easily discriminable to birds than other viewers (Maan and Cummings 2012). We have also shown that sexually selected changes on signal brightness within a single population can lead to concomitant changes in another signal attribute (hue/color). This phenomenon may lead to macro-level effects on the aposematic signal, with larger incremental changes in aposematic

brightness leading to hue changes that are observable to predators. Hence, signal brightness within *O. pumilio* populations may be shaped by sexual selection, with limited opportunity for natural selection to influence this trait due to predator sensory constraints. Taken together, our study suggests that sexual selection may generate the direction and micro-tuning of aposematic trait evolution in some populations of this species while natural selection acts as a purifying agent at coarser scales.

1.5 TABLES

	Inherent Contrast	Bird		Snake		Crab		Frog	
		ΔL	ΔS	ΔL	ΔS	ΔL	ΔS	ΔL	ΔS
Leaf 	Bright: 0.278 Dull: 0.147	Bright: 0.995(48) Dull: 0.302(0) 0.650	Bright: 1.514(100) Dull: 1.848(100)	Bright: 4.395(98) Dull: -0.303(33) 3.507	Bright: 0.712(17) Dull: 1.190(67)	Bright: 2.164(94) Dull: -0.118(8) 2.441	Bright: 1.821(92) Dull: 3.129(98)	Bright: 2.859(98) Dull: 0.461(36) 2.371	Bright: 2.756(100) Dull: 4.071(100)
Moss 	Bright: 0.731 Dull: 0.670	Bright: 3.974(100) Dull: 3.281(100) 0.542	Bright: 1.787(100) Dull: 1.898(100)	Bright: 22.746(100) Dull: 18.048(100) 3.460	Bright: 1.979(100) Dull: 1.596(94)	Bright: 11.984(100) Dull: 9.703(100) 2.196	Bright: 2.212(89) Dull: 1.249(63)	Bright: 12.843(100) Dull: 10.445(100) 2.249	Bright: 1.105 (61) Dull: 2.311(91)
Tree 	Bright: 0.618 Dull: 0.539	Bright: 3.444(100) Dull: 2.751(100) 0.600	Bright: 1.176(86) Dull: 1.421(94)	Bright: 20.623(100) Dull: 15.925(100) 3.452	Bright: 0.959(34) Dull: 1.051(63)	Bright: 10.069(100) Dull: 7.788(100) 2.345	Bright: 0.536(25) Dull: 1.772(80)	Bright: 11.188(100) Dull: 8.791(100) 2.368	Bright: 2.116 (95) Dull: 3.428(100)
Leaf Litter 	Bright: 0.243 Dull: 0.129	Bright: 1.380(95) Dull: 0.687(9) 0.678	Bright: 1.340(95) Dull: 1.701(97)	Bright: 7.594(100) Dull: 2.896(75) 3.512	Bright: 1.240(81) Dull: 1.760(97)	Bright: 3.248(100) Dull: 0.966(47) 2.441	Bright: 3.001(100) Dull: 4.308(100)	Bright: 4.156(100) Dull: 1.759(70) 2.375	Bright: 3.332 (100) Dull: 4.647(100)
Palm 	Bright: 0.237 Dull: 0.122	Bright: 1.085(61) Dull: 0.392(0) 0.666	Bright: 1.176(83) Dull: 1.538(94)	Bright: 5.626(100) Dull: 0.927(48) 3.510	Bright: 0.840(30) Dull: 1.342(81)	Bright: 2.526(98) Dull: 0.245(20) 2.441	Bright: 1.994(95) Dull: 3.302(100)	Bright: 3.247(100) Dull: 0.850(45) 2.373	Bright: 2.729 (100) Dull: 4.044 (100)
Soil 	Bright: 0.786 Dull: 0.737	Bright: 5.213(100) Dull: 4.520(100) 0.558	Bright: 1.424(100) Dull: 1.573(100)	Bright: 31.915(100) Dull: 27.217(100) 3.441	Bright: 2.227(100) Dull: 2.172(100)	Bright: 15.555(100) Dull: 13.274(100) 2.282	Bright: 0.416(20) Dull: 1.503(69)	Bright: 17.094(100) Dull: 14.696(100) 2.367	Bright: 2.118(95) Dull: 3.429(100)

Table 1.1: Median contrast estimates for three putative predator visual systems and conspecifics. Values in table cells are calculated separately for bright (> mean log of the total reflectance flux) and dull (< mean log of the total reflectance flux) male classes. Adjacent values in parentheses indicate the percentage of individuals that exceeded the signal detection threshold (1.0) for each class. Underlined values do not exceed the signal-noise detection threshold. Cells that are shaded (light blue) are those where the values for the two male brightness classes are likely to be indistinguishable (difference <1, as indicated by Wilcoxon rank sum tests). Values along the bottoms of the cells contained in circular gray boxes represent the median Euclidian distance taken from pairwise contrast matrixes for all possible male pairings.

	Bird 	Snake 	Crab 	Frog 
	Overall Conspicuousness	Overall Conspicuousness	Overall Conspicuousness	Overall Conspicuousness
Leaf 	Bright: 1.861(100) Dull: 1.878(100)	Bright: 4.511(98) Dull: 2.286(92)	Bright: 3.017(100) Dull: 3.278(100)	Bright: 4.097(100) Dull: 4.168(100)
Moss 	Bright: 4.368(100) Dull: 3.812(100)	Bright: 22.803(100) Dull: 18.133(100)	Bright: 12.166(100) Dull: 9.783(100)	Bright: 12.991(100) Dull: 10.859(100)
Tree 	Bright: 3.656(100) Dull: 3.097(100)	Bright: 20.650(100) Dull: 15.962(100)	Bright: 10.119(100) Dull: 8.137(100)	Bright: 11.490(100) Dull: 9.549(100)
Litter 	Bright: 1.950(100) Dull: 1.815(100)	Bright: 7.755(100) Dull: 3.950(100)	Bright: 4.559(100) Dull: 4.534(100)	Bright: 5.416(100) Dull: 4.961(100)
Palm 	Bright: 1.634(100) Dull: 1.581(98)	Bright: 5.746(100) Dull: 2.714(98)	Bright: 3.383(100) Dull: 3.373(100)	Bright: 4.338(100) Dull: 4.230(100)
Soil 	Bright: 5.424(100) Dull: 4.797(100)	Bright: 31.985(100) Dull: 27.300(100)	Bright: 15.598(100) Dull: 13.385(100)	Bright: 17.332(100) Dull: 15.206(100)

Table 1.2: Median "overall conspicuousness" contrast estimates for three putative predator visual systems and conspecifics. Values are calculated separately for bright (> mean log of the total reflectance flux) and dull (< mean log of the total reflectance flux) male classes. Adjacent values in parentheses indicate the percentage of individuals that exceeded the signal detection threshold for each class. Cells that are shaded (light blue) are those where the values for the two male brightness classes are indistinguishable (difference <1, as indicated by Wilcoxon rank sum tests).

1.6 FIGURES

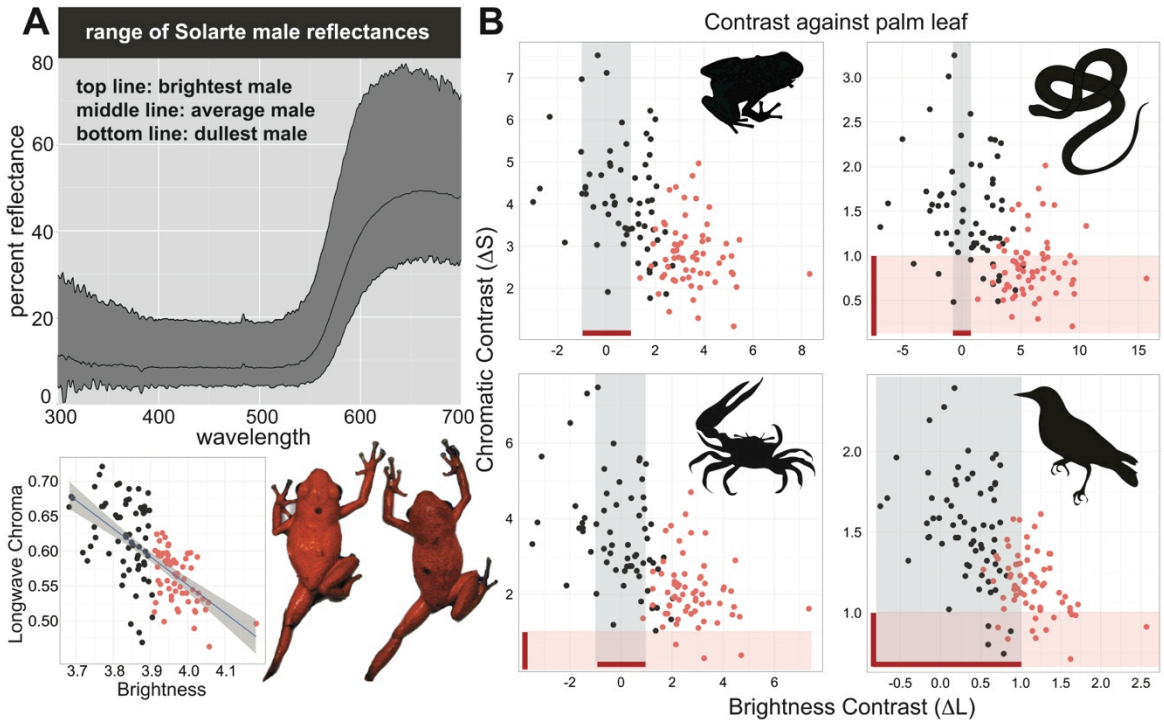

Figure 1.1: Perceptually unbiased (A) and taxon-specific estimates (B) of color and brightness. (A) Variation in reflectance across different wavelengths of Solarte males. Dark region of line plot represents the observed range of Solarte male reflectances. The scatterplot represents the relationship between two perceptually unbiased measures: brightness (log of the total reflectance flux) and long-wave chroma, the proportion of long-wave (600-700nm) reflectance relative to the total. The accompanying photographs depict two male frogs representing some of the phenotypic variation observed in the population. (B) Brightness contrast plotted against chromatic contrast against a dry palm leaf background. For all scatterplots, dots are color-coded according to perceptually unbiased reflectance flux brightness classes (brighter than average = red, duller than average = black). Shaded areas of the plots indicate regions of perceptual contrast space in which individuals are likely to be indiscriminable from the background in that particular channel (gray=brightness contrast; red=chromatic contrast; values are below the signal detection threshold, <1.0).

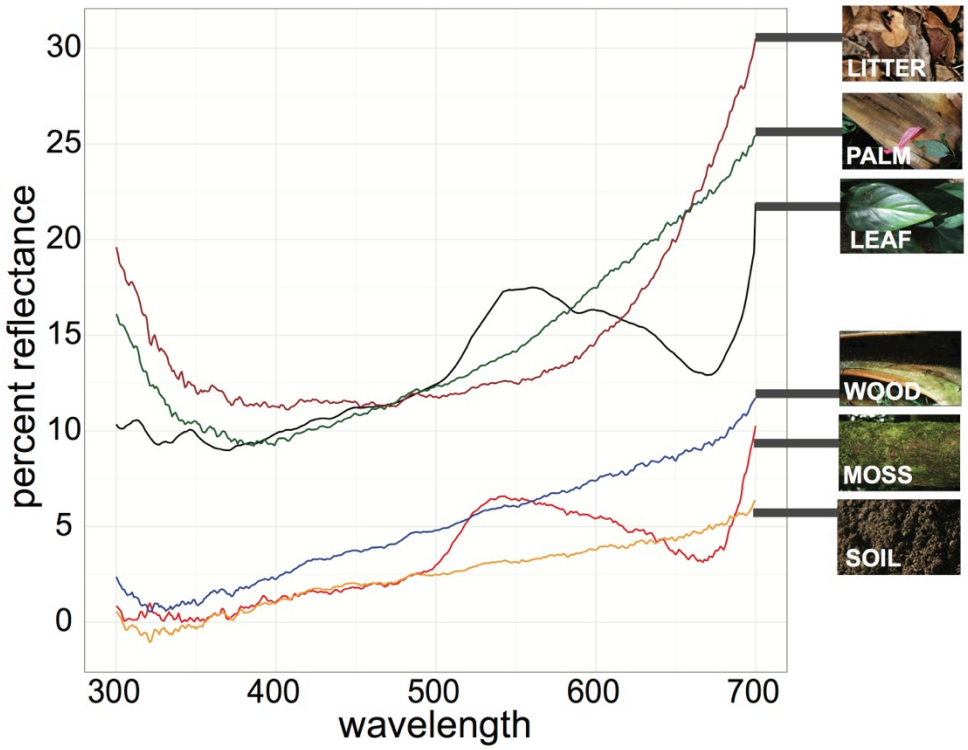

Figure 1.2: Reflectance spectra of the six substrates used in analyses.

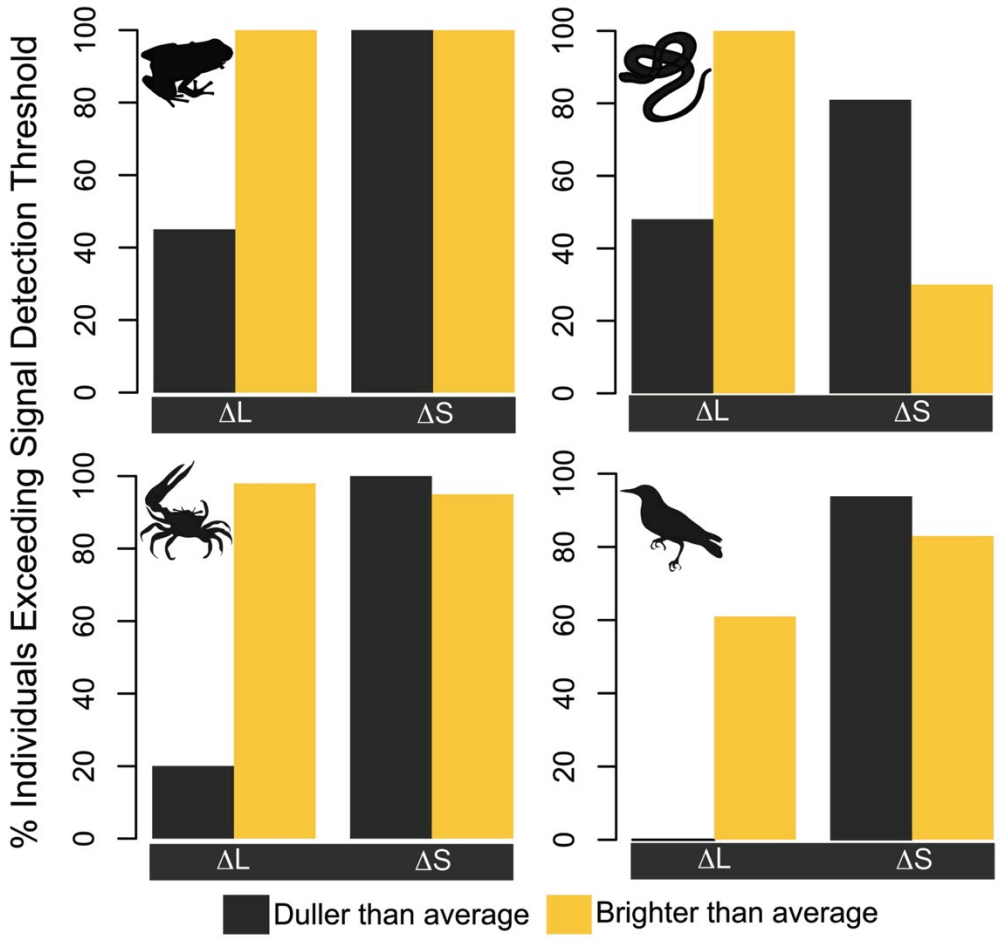

Figure 1.3: Brightness (ΔL) and chromatic (ΔS) contrasts for the four taxon-specific visual models against a palm leaf background. Bar plots indicate the percentage of individuals that exceeded the signal detection threshold for the two different male inherent brightness classes (dark bars = duller than average reflectance flux category; light orange bars = brighter than average reflectance flux category).

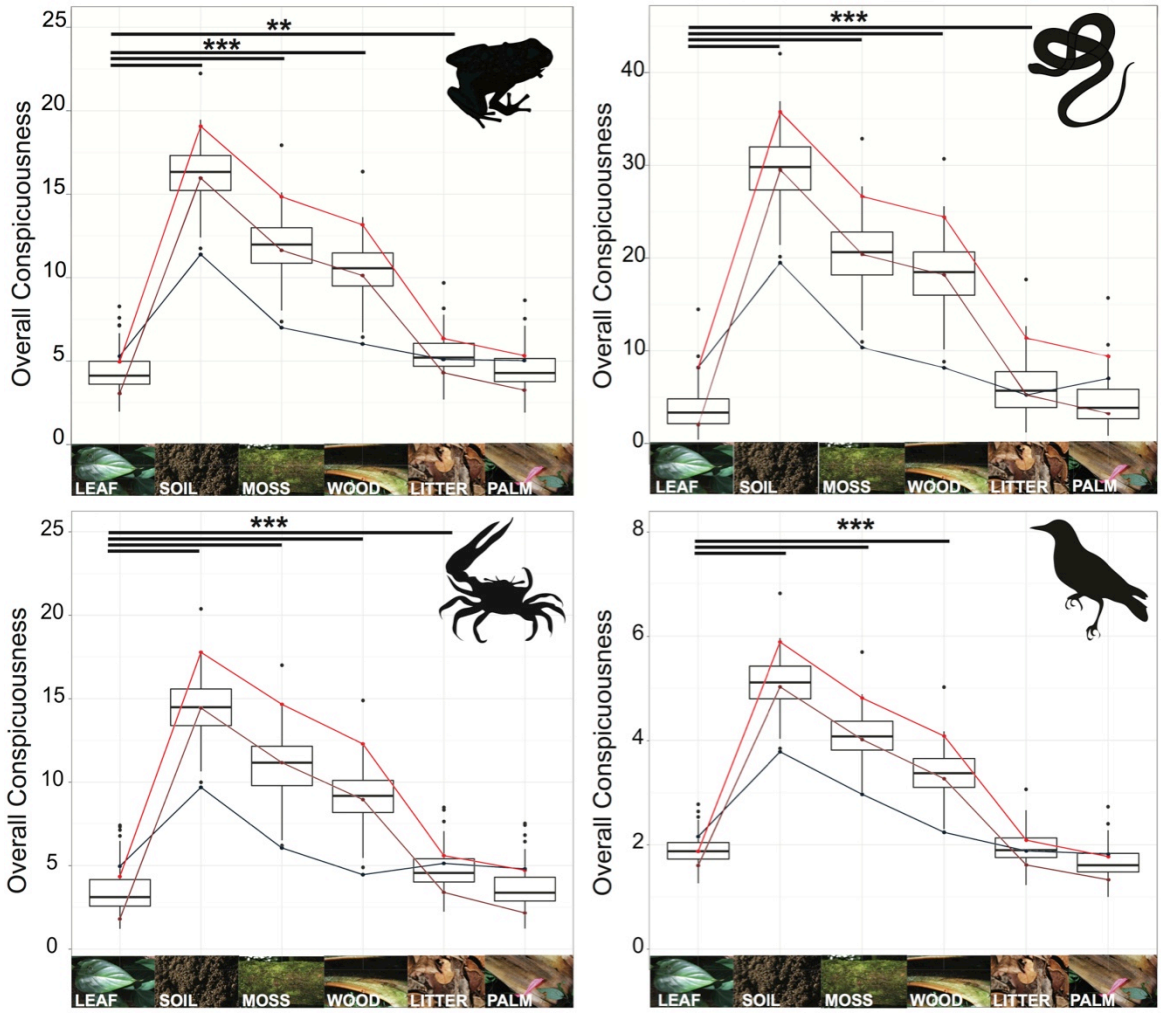

Figure 1.4: Influence of signal background on conspicuousness for frog, snake (trichromatic), crab (dichromatic) and avian (tetrachromatic) visual systems. Box-and-whisker plots represent overall conspicuousness estimates for all males in dataset. Boxes span the 1st and 3rd quartile of the data, and horizontal black lines represent the median. Whiskers span the range of the data, excepting outliers (black dots). Overlaid lines indicate the overall conspicuousness estimates for 3 individual frogs (red line = exceptionally bright male, brown line = average male, black line = exceptionally dull male) on the different substrate backgrounds. Asterisks indicate whether relationships were statistically significant (** = $P < 0.01$, *** = $P < 0.001$). Please note that y-axes of the plots are at different scales.

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CHAPTER 2:

Aposematic signal variation predicts male-male interactions in a polytypic poison frog

ABSTRACT

Many species use conspicuous "aposematic" signals to communicate unpalatability/unprofitability to potential predators. Although aposematic traits are generally considered to be classic examples of evolution by natural selection, they can also function in the context of sexual selection, and therefore comprise exceptional systems for understanding how conspicuous signals evolve under multifarious selection. We used males from a highly territorial poison frog species in a dichotomous choice behavioral test to conduct the first examination of how aposematic signal variation influences male–male interactions. Our results reveal two behavioral patterns: *(1)* male dorsal brightness influences the behaviors of male conspecifics such that males approach and call to brighter males more frequently, and *(2)* a male's dorsal brightness predicts his own behavior such that bright males approach stimulus frogs faster, direct more calls to bright stimulus frogs, and exhibit lower advertising call pulse rates (a fitness-related trait). These findings indicate the potential for sexual selection by male–male competition to impact aposematic signal evolution.

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2.1 INTRODUCTION

Many species use conspicuous 'aposematic' signals to communicate unpalatability to potential predators, a strategy that is widespread throughout the animal kingdom (Ruxton et al. 2004). While aposematic traits are generally considered to be classic examples of evolution by natural selection (Müller 1879), they can also function in the context of conspecific communication (Summers et al. 1999; Jiggins et al. 2001). Aposematic organisms therefore comprise exceptional systems for understanding how conspicuous traits evolve under multifarious selection. However, several potentially important selective forces remain unexplored in aposematic systems. For example, aposematic traits might influence the intensity and/or outcomes of aggressive interactions between male conspecifics. This is especially probable given the well-documented role that conspicuous, non-aposematic signals play in male territorial behaviors (Andersson 1994). Male brightness/coloration is one conspicuous signal that males may attend to during territorial interactions in aposematic species. Studying the presence or absence of male selection on warning coloration will elucidate the potential for evolutionary feedbacks between intrasexual selection (e.g., male-male competition) and other forms of selection on aposematic signals.

Many frogs of the family Dendrobatidae are aposematic, exhibiting bright coloration and patterning and sequestering toxins acquired from their diet (Santos et al. 2003). The strawberry poison frog, *Oophaga [Dendrobates] pumilio*), is perhaps the most polytypic of the poison frog species. *O. pumilio* is monotypic in coloration across most of its range from Nicaragua to Panama, but in the Bocas del Toro archipelago in western Panama the species exhibits dramatic variation in both hue and brightness across island populations and on the mainland (Daly and Myers 1967; Siddiqi et al. 2004). The selective forces that have produced this remarkable variation remain unclear. *O. pumilio* has the potential for color vision (Siddiqi et al. 2004) and there is evidence of sexual selection by female preference on male coloration and brightness in this species (Summers et al. 1999; Reynolds and Fitzpatrick 2007; Maan and Cummings 2008, 2009). Sexual selection by female choice on ecologically important traits can result in sexual dimorphism in those traits (Lande and Arnold 1985). Coincident

with theory, one population of *O. pumilio* contains males that are significantly brighter than females (Maan and Cummings 2009).

Although past studies indicate the potential for female preferences to drive color variation in *O. pumilio*, the other major component of sexual selection, male-male competition, has not yet been investigated. It has been hypothesized that male secondary sexual characteristics can originate through male-male competitive interactions (Berglund et al. 1996). This may be particularly true for highly territorial species such as *O. pumilio*. Male *O. pumilio* exhibit territory site fidelity (McVey et al. 1981) and vigorously defend their sites through vocalizations and close-range aggressive encounters (Bunnell 1973; Forester et al. 1993; Baugh and Forester 1994; Gardner and Graves 2005; Pröhl 2005). Thus, the selective pressures imparted by male-male competition may conflict with or facilitate signal divergence mediated by predators or female preference across populations.

Given the evidence for female preference for brighter males in some populations of this species, we predicted that male-male interactions could also be mediated by male brightness. We examined if and how aposematic signal variation affects male-male interactions in *O. pumilio* by experimentally manipulating the brightness of stimulus males and recording the responses of focal males, and assessing whether the brightness of stimulus males and/or focal males predicted the outcomes of male interactions. Here we report that a male's brightness both robustly predicts his own behavior and influences the behavior of competitors. Together, these findings indicate that male intrasexual selection may serve as a mechanism to affect color variation in *O. pumilio*.

2.2 METHODS

Animals

Male *O. pumilio* (N = 75) were captured during daytime hours during July and August of 2009 and kept at the Bocas del Toro Field Station of the Smithsonian Tropical Research Institute, Panama. We used frogs from one population (Isla Solarte) in which females have previously been shown to prefer to interact with brighter males and in which there is sexual dimorphism, as well as intrasexual variation, in dorsal brightness (Maan and Cummings

2009). Male frogs were located in the field; if they were calling (N = 25), their calls were recorded for a minimum of 1 minute using a Marantz PMD660 portable digital recorder (Marantz, Mahwah, NJ). Frogs were then captured and measured for snout-vent length (SVL: to the nearest 0.1 mm), mass (to the nearest 0.01 g), body temperature at the dorsal surface (within 0.1C), and spectral reflectance in a temperature-controlled room (~23C) within 24 hours of capture. Body temperature was measured using an infrared laser thermometer (Mastercool, Randolph NJ) immediately prior to spectral reflectance measurements for a majority of the frogs tested in behavioral assays (N = 57) because it can induce facultative color change in other amphibians (Tattersall et al. 2006) and is associated with fitness in ectotherms (Huey and Kingsolver 1989). Frogs were housed individually in outdoor terraria, fed a diet of termites, ants, and fruit flies, with fresh water provided twice daily.

Male Coloration

Spectral reflectance measurements were taken of each male at the head, dorsum, belly, and throat (2 measurements per region) using a EPP200C UV-VIS spectrometer, SL-4 Xenon lamp, and R400-7 reflectance probe (StellarNet Inc., Tampa, FL). Spectralon white standard measurements were taken between frogs to account for lamp drift.

Call Analysis

Male calls were edited for length and background noise in Audacity software and analyzed for call characteristics in Raven software. The call characteristics analyzed included mean call rate, mean call duration, duty cycle (mean call rate x mean call duration), pulse rate (number of pulses per call segment), and dominant frequency, as described by Pröhl (2003). Call characteristics were scored independently by two observers and averaged.

Experimental Treatments and Setup

Rival Choice Experiments

Focal male responses towards brighter and duller stimulus males were evaluated in a series of two-way choice experiments modified from those used previously in our laboratory

(Fig 2.1a; Maan and Cummings 2009). Focal frogs were presented with two stimulus males originating from the focal frogs' native population. Stimulus male pairs were matched for size, mass, and dorsal reflectance spectra (all differences between males were within one standard deviation of the population mean). The setup was similar to previous experiments (Maan and Cummings 2009) and consisted of three boxes of UV-transparent clear acrylic, one focal male chamber (40 x 20 x 20 cm) and two stimulus male chambers (half circles with a radius of 10 cm and 20 cm high). The visual background of each stimulus male chamber was black to minimize differences in color contrast between the stimuli, and a visual barrier blocked the stimulus males' view of each other. Experiments were carried out in an illumination-controlled room, and both stimulus males were illuminated with light that mimics conditions on the forest floor, using one 22 inch UV 20W bulb and one 100W "Daylight Blue" incandescent bulb, filtered by two green-blue filters (Lee 728, CyanGel 4315).

We manipulated focal male perception of stimulus male brightness by using neutral density filters (one Lee 298 and one GamColor 1514) above one stimulus male for the first 10 minutes of observation. For the next 10 minutes, these filters were moved to the other male's chamber, thus reversing the brightness difference between the stimulus males. We reduced male brightness by ~65%, within 2.5 standard deviations of mean male brightness in the Solarte population. Irradiances for both the dull and bright lighting conditions fell within the upper quartile of territory irradiance flux ($\log(\sum I(\lambda)$ from 300-700nm)) in this population (M.E. Cummings and M.E. Maan, *unpublished data*). Control experiments used identical light treatments but with empty male chambers to control for a possible focal male tendency to approach darker or brighter areas.

No focal male was tested more than twice per day, and stimulus pairs were used for a maximum of eight experiments per day. All frogs were tested within one week of capture. Focal males were acclimated to the experimental chambers for at least 60 minutes and allowed to interact freely with a native female within their chamber to motivate territorial behavior. Stimulus males were acclimated for 30 minutes without visual contact. After acclimation, visual barriers were removed and the focal male was placed under a glass in the

middle of his chamber to allow observation of both stimulus males for 2 minutes. The glass was then lifted and the focal male was observed for two 10-minute periods.

Territorial interactions in *O. pumilio* involve approach by the intruder male and impingement on or near a male's territory followed by a stereotyped series of behaviors, including calling and approaches (L.R. Crothers *pers. obs;* Bunnell 1973; Baugh and Forester 1994; Gardner and Graves 2005; Pröhl 2005), that can either lead to escalation (physical contact involving chases and wrestling) or submission by the intruder male and subsequent exit from the male's territory. Thus, to score male behaviors we defined an "interaction zone" as the area within 4cm (2 body lengths) of each stimulus male, as done previously (Maan and Cummings 2008, 2009). In each trial we recorded the focal male's latency to approach an interaction zone, time spent in the interaction zone with each stimulus male, the number of times that focal males approached each stimulus male, as well as the number of calls to each stimulus male. After 10 minutes of observation, visual barriers were inserted again, positions of neutral density filters were reversed, and the focal male was confined under a glass for 2 minutes. After this, the barriers were removed and observations resumed. Most males were tested in both experimental and control treatments.

Analysis of Brightness

Dorsal reflectance spectra were obtained by averaging measurements of the head and dorsum (two measurements per region). To measure "brightness" differences between males, we evaluated the log of the difference in total reflectance flux [$\log(\sum R(\lambda)$ from 300-700nm)] and the estimated difference in brightness contrast (ΔL) of frog coloration when viewed against a natural background by a *O. pumilio* viewer using a receptor-based visual model described previously (Maan and Cummings 2009).

Data Analysis

All statistical tests were performed in R software. Count data (approaches, calls) were summed across the two trials for each male, and data from all males were used in these analyses (N = 75 in experimental trials, N = 71 in control trials). Focal frog approach and

call count data were analyzed using Wilcoxon signed-rank tests to accommodate data that were not normally distributed. Latency analyses were restricted to males that entered the interaction zone in both trials (57 males in experimental trials, 52 in controls). Latencies were averaged across trials for each included male. Five males did not approach the interaction zone in either trial. Total focal frog approach latency and interaction time data were modeled using generalized linear models (GLM) with underlying Poisson or quasi-Poisson distributions, and proportions of focal frog approach, call and interaction time with the different stimuli using a Binomial or quasi-Binomial distribution to adjust for non-normality and underdispersion/overdispersion of data. Individuals that did not approach the interaction zone or call were not included in proportion analyses. A Chi-square goodness-of-fit test was used to assess whether focal males exhibited a bias in the first stimulus male that they approached.

First, we tested whether focal males exhibited differential responses towards the more brightly- or darkly-illuminated stimulus males (experimental trials) or empty chamber (controls). Subsequently, we evaluated whether focal male behavior was predicted by a male's own brightness. We also tested whether male brightness predicted advertisement call characteristics, body size, mass, or body temperature using linear models.

2.3 RESULTS

Focal Male Preferences for Brighter Stimulus Males

Focal males approached (Fig 2.1b; V = 1139, P = 0.011, N = 75) and called to (Fig 2.1c; V = 271.5, P = 0.014, N = 75) the more brightly illuminated stimulus male significantly more often than the dull stimulus male. Focal males did not exhibit biases in the first frog that they approached (χ^2 = 0.5, P = 0.480), or interaction time with bright versus dull stimuli (V = 1546, P = 0.076, N = 75; mean = 400.9 sec for bright chambers, 308.4 sec for dull chambers). Males showed no bias in the number of approaches (Fig 2.1b; V = 506, P = 0.698, N = 71) or time spent (V = 1111, P = 0.864, N = 71; mean = 346.6 sec for bright

chambers, 349.9 sec for dull chambers) in front of empty chambers with differing illumination during control trials. No males called during control trials (Fig 2.1c).

Brightness of Focal Males and Focal Male Behavior

Brighter focal males approached stimulus males faster than duller focal males (*total reflectance*: F = 5.936, P = 0.018, N = 57; (shown in Fig 2.2a); Δ*L*: F = 10.166, P = 0.002). This tendency remained even when an exceptionally bright focal male was removed from the analysis (*total reflectance*: F = 4.333, P = 0.042; Δ*L*: F = 8.006, P = 0.007, N = 56). Focal male brightness did not predict latency to approach control chambers (*total reflectance:* F = 2.109, P = 0.153; Δ*L*: F = 2.140, P = 0.150, N = 52).

Brighter focal males directed a greater proportion of their calls to the bright stimulus frog than did duller focal males (Fig 2.2b; *total reflectance:* Z = 2.140, P = 0.032; Δ*L*: Z = 2.182, P = 0.029, N = 29); this tendency remained even when the brightest male was removed from the analysis (*total reflectance:* Z = 2.588, P = 0.010; Δ*L*: Z = 2.679, P = 0.007, N = 28). However, focal frog brightness did not predict the proportion of approaches to (*total reflectance:* Z = 0.066, P = 0.948; Δ*L*: Z = 0.132, P = 0.895, N = 69) or the proportion of time spent with the brighter stimulus frog (*total reflectance:* F = 0.4357, P = 0.512; Δ*L*: F= 0.634, P = 0.429, N = 70). No relationships were detected between focal male brightness and the total number of approaches (*total reflectance:* Z = -0.568, P = 0.570; Δ*L*: Z = 0.176, P = 0.860, N = 75), calls (*total reflectance*: Z = -0.400, P = 0.689; Δ*L*: Z =-1.096, P = 0.273, N = 75) or total interaction time spent with stimuli (*total reflectance:* F = 0.1006, P = 0.752; Δ*L*: F = 0.0038, P = 0.951, N = 75).

A negative relationship was observed between focal male brightness and body temperature (Fig 2.2c; *total reflectance:* F = 5.1423, P = 0.027, N = 60), though this result was not significant using brightness estimates calculated with the frog visual model (Δ*L*: F = 3.003, P = 0.088). Brightness was not correlated with mass (*total reflectance:* F= 0.9225, P = 0.34; Δ*L*: F= 0.6019, P = 0.440, N = 75), SVL (*total reflectance:* F =1.3123, P = 0.256; Δ*L*: F = 1.6451, P = 0.204, N =75), or SVL-mass residuals, a common measure of body condition (*total reflectance:* F = 0.082, P = 0.776; Δ*L*: F = 0.0037, P = 0.952, N = 75). Finally, we found

that brighter males exhibited lower advertisement call pulse rates in the field than duller males (Fig 2.2d; *total reflectance:* $F = 5.0684$, $P = 0.034$; ΔL: $F = 5.2791$, $P = 0.031$, $N = 25$). Ambient temperature did not account for differences in call pulse rates ($F = 0.0615$, $P = 0.807$, $N = 20$). Brightness was not predictive of mean call rate, mean call duration, duty cycle, or dominant frequency (data not shown).

2.4 DISCUSSION

Our agonistic choice experiments demonstrate that males of at least one of the polytypic *O. pumilio* populations attend to the brightness of potential rivals during male-male interactions. Brighter stimulus males elicited significantly more calls and approaches from focal males than did their dull counterparts (Fig 2.1b,c). Both of these behaviors are central components of aggressive interactions between males in the field (*pers. obs*; Bunnell 1973; Gardner and Graves 2005; Pröhl 2005), and the finding that both bright and dull males respond to a potential rival's brightness suggests a population-wide response to this cue. It is possible that brighter males were approached and called to more often in experimental trials by virtue of their enhanced conspicuousness. However, our findings are unlikely to result from simple differences in the detectabilities of bright versus dull males, as focal frogs neither approached bright males first in behavioral tests, nor spent more time interacting with them. Furthermore, no simple preferences for brightly illuminated chambers were observed, as we found no biases in male behaviors directed at empty chambers with differing illumination.

While focal males responded significantly more towards brighter potential intruders than duller ones, the nature of the response differed depending upon the focal male's own brightness. We found that focal male brightness predicted focal male behavior, with brighter males approaching stimulus frogs faster than their dull counterparts and directing more of their calls to bright stimulus frogs (Fig 2.2a,b). Other physiological attributes that correlate with brightness may underlie the behavioral differences among males of differing brightness. For instance, the correlation between male body temperature and brightness (Fig 2.2c) might

indicate that brighter males are in better condition (Huey and Kingsolver 1989), and therefore able to respond more aggressively. This correlation is unlikely to result from simple changes in ambient temperature during the course of our experiments, since *1)* measurements were performed in a temperature controlled room, and *2)* previous investigators have not found temperature effects on *O. pumilio* coloration (Summers et al. 2003). Further investigation into mechanisms underlying color variation in this species will prove especially informative.

Interestingly, brighter males exhibit a lower call pulse rate than duller males (Fig. 2.2d), which several lines of evidence indicate may likewise be a fitness-related trait. Vocalizations have reliably predicted outcomes of territorial disputes in *O. pumilio* and related species (Stewart and Rand 1991; Baugh and Forester 1994), and a previous study (Pröhl 2003) indicates that pulse rate correlates negatively with mating success in this species. Thus, we provide indirect evidence that bright males exhibit call characteristics that may have fitness consequences in the field.

In species in which males provide some parental care, sexually selected traits (such as male brightness is in this population) are predicted to be condition-dependent, functioning as honest indicators of an individual's condition/quality (Andersson 1986). Advertising conspicuously with enhanced brightness may enhance mating success but only in individuals that have the energetic reserves necessary to bear the costs of the signal (Price 2006). As calling alone is energetically expensive in many anuran taxa (Navas et al. 2008) frequent territorial interactions between adjacent males could impose fitness costs, even if interactions do not escalate to full contact. Taken together, our behavioral (latency response and call pulse rate) and physiological data (correlation between brightness and body temperature) suggest that male brightness may thus function as an indicator trait in poison frogs.

There is ample evidence for bright ornamentation functioning as a badge of status in many taxa (Andersson 1994; Pärt and Qvarnström 1997; Korzan and Fernald 2006), and orange and red coloration (as exhibited by the Solarte population) is well documented as a common signal of dominance and aggression (Pryke 2009). Males in this population could thus use brightness to assess rival territorial abilities (Berglund et al. 1996). Since males vary greatly in brightness within this population (mean ΔL= 15.8927; SD = 2.1580), the

differences we observed in male behavior may have perceptible consequences in the field. If focal male behaviors predict defensive capabilities in the field, with brighter males having greater defense of their territories, then male-male interactions may be acting additively or synergistically with female preference to promote enhanced male brightness in this population. Alternatively, if eliciting greater response from rivals incurs fitness costs for bright males, male-male interactions could act in opposition to female mating preferences and constrain male brightness evolution. Our results cannot distinguish between these or alternative scenarios in which male competitive interactions may affect the direction of aposematic signal evolution, but future studies will address the fitness consequences of *O. pumilio* male response to rival brightness.

In conclusion, our behavioral results suggest that there is a third component to the evolution of aposematic signals in this species— the response of *males* to signal variation. Male *O. pumilio* respond to the brightness of potential rivals, preferentially approaching and calling to bright stimulus males, and brighter males approach potential rivals faster and direct proportionally more calls to brighter rivals. Previous work has shown that predators and potential mates attend to coloration (Summers et al. 1999; Jiggins et al. 2001; Ruxton et al. 2004) and brightness in aposematic species (Prudic et al. 2007; Maan and Cummings 2009), and here we demonstrate that male rivals also respond differentially to signal variation. Since territorial interactions between males within the Solarte population are common (L.R. Crothers, *pers. obs.*), differential territorial responses based on rival brightness may be a significant component of signal selection. Females in this population prefer to interact with bright males and males are significantly brighter than females (Maan and Cummings 2009); whether differential responses of males towards brighter potential rivals acts in parallel or in conflict with female-mediated color divergence depends on the outcomes of these interactions in the field. Together, our data provide the first evidence of males using an aposematic signal (brightness) as a cue during territorial behaviors, and tantalizing evidence that brightness may be a conditional signal in these populations. Ongoing studies in the field will further elucidate the roles that male-male competition plays in the evolution of aposematic signals within these populations, and clarify how multiple agents of selection contribute to signal evolution within this species.

2.5 FIGURES

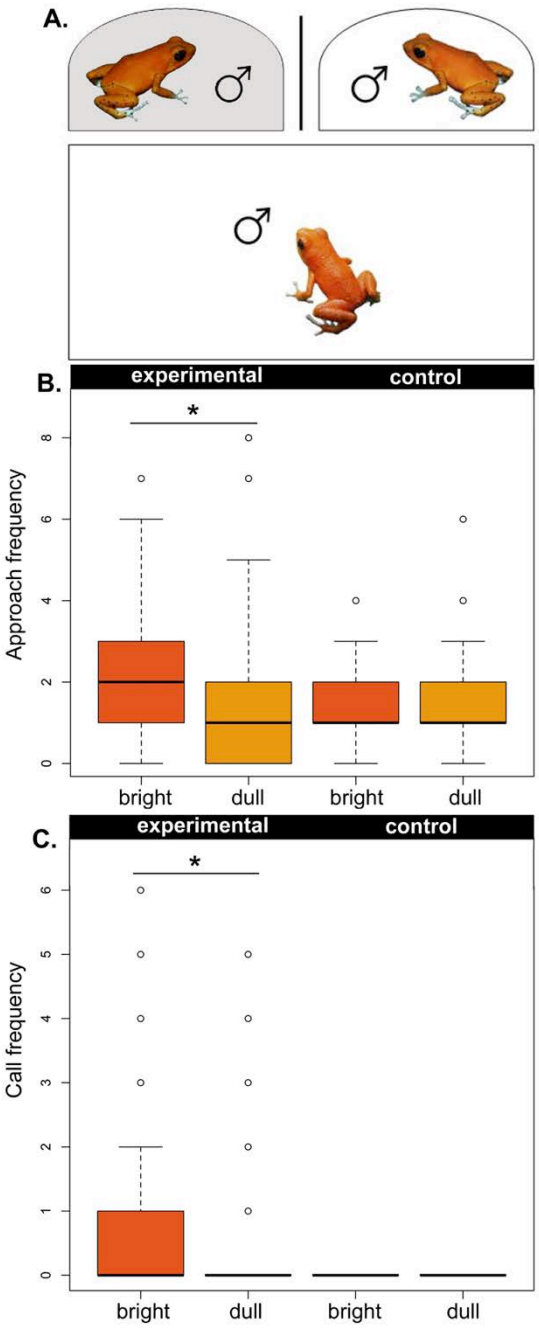

Figure 2.1 :

Behavioral responses of focal males to stimulus males. (A) Schematic of agonistic choice experimental setup showing two stimulus males (small compartments) and the focal male (large compartment). Box-and-whisker plots of (B) experimental and control trial approach frequencies of focal males to bright and dull chambers, and (C) experimental and control trial call frequencies of focal males to bright and dull chambers. Boxes span the 1st and 3rd quartile of the data, and horizontal black lines represent the median. Whiskers span the range of the data, excepting outliers (open circles).

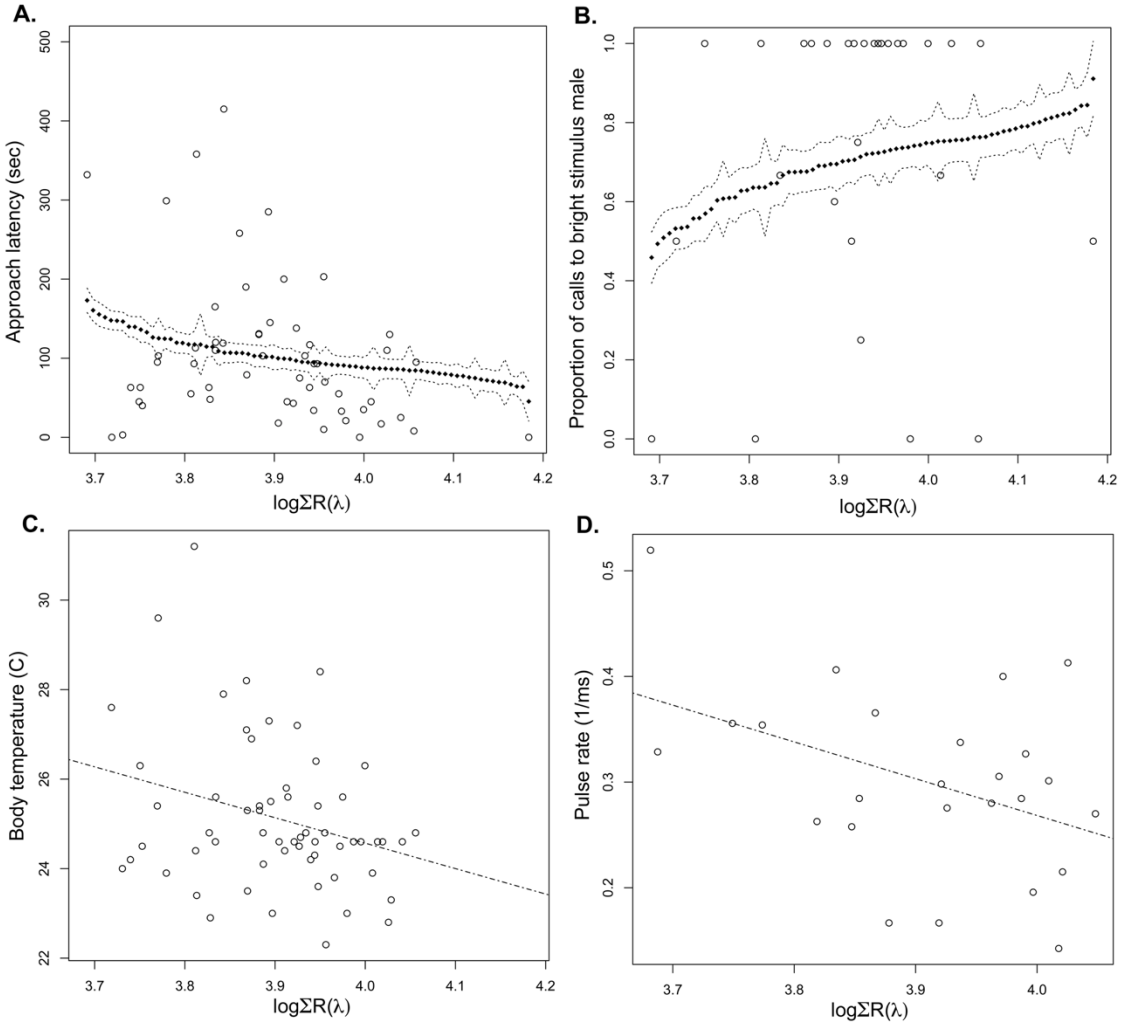

Figure 2.2: Relationships to focal male brightness (log(∑R(λ)). Open circles represent data points for all panels. (A) Latency for focal male to approach interaction zone. Solid diamonds represent predicted probability values of the "quasi-Poisson" GLM. Dotted lines flanking the predicted values represent the standard error. (B) Proportion of calls directed to bright stimulus male by focal male. Solid diamonds represent predicted probability values of the binomial GLM. Dotted lines flanking the predicted values represent the standard error. (C) Body temperature at dorsal surface for males at time of reflectance measurements. Dotted line represents best-fit line predicted by linear model (Multiple R^2: 0.081, Adjusted R^2: 0.066). (D) Call pulse rate of males in the field. Dotted line represents best-fit line predicted by linear model (Multiple R^2: 0.181, Adjusted R^2: 0.145).

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CHAPTER 3:

A multifunctional warning signal behaves as an agonistic signal in a poison frog

ABSTRACT

Aposematic species use conspicuous "warning" signals to communicate unprofitability to potential predators. Although warning signals are classic examples of communication systems that evolved by natural selection, they can also function in the context of sexual communication and are therefore particularly useful for investigating conspicuous trait evolution under multifarious selection. To test whether aposematic signals also serve to mediate intrasexual disputes, we observed males from a highly territorial poison frog species (*Oophaga pumilio*) in their native territories and in experimental dyadic contests to assess the influences of body characteristics such as warning signal brightness and body size on the outcomes of territorial interactions. We report here that while neither male size (snout-vent length) nor mass significantly predicted male aggressiveness (latency to call) in dyadic contests, a male's dorsal brightness was a significant predictor of willingness to initiate aggressive interactions, with brighter males exhibiting a shorter latency to call than duller males. Furthermore, brightness asymmetries between males predicted the outcomes of contests such that asymmetries were smaller in escalated aggression trials (where both males called), and brighter males were more likely to be the sole aggressor in trials with large asymmetries. These tests, combined with previous work, provide evidence that warning coloration has been co-opted as an agonistic indicator trait in this aposematic amphibian, and reveal the potential evolutionary lability of conspicuous traits that arise through natural selection.

***This chapter is currently in review as Crothers & Cummings. Behavioral Ecology.**

3.1 INTRODUCTION

Humans have admired and puzzled over conspicuous animal signals for millennia (Darwin 1871; Ovid 2008). The expression of these traits typically represents a compromise between the forces of natural selection and sexual selection (Darwin 1871; Darwin 1887; Endler 1983). While sexual selection often drives the evolution of conspicuous traits (Andersson 1994), they can also evolve via the process of natural selection. In aposematic species, conspicuous signals such as bright "warning coloration" have evolved to communicate some form of unprofitability to predators (Wallace 1867; Ruxton et al. 2004). By virtue of their conspicuousness, warning signals may often be co-opted for use in other scenarios, such as intraspecific communication. Thus, although predators have historically been thought of as the primary agents shaping the evolution of aposematic signals (Müller 1879), evidence suggests these signals can function in the context of mate selection (Jiggins et al. 2001; Nokelainen et al. 2012), and that sexual selection can influence the direction of aposematic trait evolution (Maan and Cummings 2009). Conspicuous sexual signals often serve as traits of dual utility, used in mate choice and in male contests (Berglund et al. 1996). However, despite a rapidly growing body of evidence that conspecifics pay attention to aposematic coloration in potential mates (Summers et al. 1999; Jiggins et al. 2001; Maan and Cummings 2008; Nokelainen et al. 2012), investigations into the influence of male-male competition on warning signal evolution have been rare (Crothers et al. 2011; Rudh et al. 2013).

The potential for intrasexual selection to impact warning coloration is especially probable given the well-documented role that conspicuous, non-aposematic signals play in territorial behaviors (Andersson 1994; Berglund et al. 1996). While phenotypic characters used in agonistic assessment are often intrinsically linked to agonistic ability (body size: Huntingford and Turner 1987; weapons: Emlen 2008), conspicuous coloration has evolved to function as an agonistic assessment signal in some birds, lizards and insects (e.g., "badge of status" signals: Rohwer 1975; Rohwer 1982; Moller 1987; Maynard Smith et al. 1988; Johnstone and Norris 1993; Pryke et al. 2001; Tibbetts and Lindsay 2008; Hamilton et al. 2013). In species where territorial interactions are common, these identifiable agonistic

signals can allow for the assessment of rival aggressiveness or resource holding potential, and if asymmetries in these traits are perceptible to contest participants then interactions can be settled before overt aggression commences (Maynard Smith et al. 1988). Since aposematic signals are highly conspicuous, can correlate with metabolic phenotype (Santos and Cannatella 2011; Pegram et al. 2013), and may be readily co-opted as sexual signals, they represent clear candidates for agonistic indicator signals. Here, we use natural phenotypic variation in the highly territorial and aposematic strawberry poison frog (*Oophaga [Dendrobates] pumilio*) to assess the influence of bright male warning coloration on aggressiveness and on the outcomes of dyadic male contests.

The strawberry poison frog exhibits dramatic variation in hue and brightness across island populations and on the mainland of the Bocas del Toro archipelago of Panama (Daly and Myers 1967). Genetic drift has largely been ruled out as a major source of this variation (Rudh et al. 2007; Brown et al. 2010; Wang and Summers 2010); sexual selection is believed to be the major force promoting warning color diversification in this species (reviewed in Cummings and Crothers 2013; Gehara et al. 2013). Female *O. pumilio* show preferences for brighter males, and at least one population contains males that are significantly brighter than females (Solarte population; Maan and Cummings 2009). *O. pumilio* is characterized by elaborate maternal care (Summers et al. 1997) and great variance in male mating success (Pröhl and Hödl 1999). Furthermore, males of the species are highly territorial, exhibiting territory site fidelity (McVey et al. 1981), and guarding areas that contain sites for foraging, tadpole rearing, and perches for calling to females (Donnelly 1989; Pröhl 1997). Male *O. pumilio* maintain small territories, which in dense populations such as Solarte average only 2-3m^2 (Pröhl and Ostrowski 2011), and which they vigorously defend against each other through vocalizations and close-range aggressive encounters (Bunnell 1973; Forester et al. 1993; Baugh and Forester 1994; Gardner and Graves 2005; Pröhl 2005). Hence, sexual selection in this species appears to have significant intersexual and intrasexual components, and the exceptionally bright warning coloration found in males of the Solarte population appears likely to be used in competitive interactions (Crothers et al. 2011). Here, we test whether the brightness of males' warning coloration can be used as an indicator of

aggressiveness or competitive ability in this species, a possibility that until now has been unexplored in any aposematic animal.

3.2 METHODS

Territorial adult males were located in the field during daytime hours in 2010 and 2012 on Isla Solarte, in Bocas del Toro, Panama (N 09°20.014' W 82°13.197'). Males were captured and kept individually in plastic 475mL deli containers moistened with ultraviolet (UV) purified water until body measurements were taken within several hours of capture at the Smithsonian Tropical Research Institute (STRI) in Bocas del Toro, Panama.

Staged Dyadic Contests

In June and July of 2010, we conducted a series of behavioral tests in the field to elucidate the intrinsic competitive abilities of males of varying brightness. 110 calling males were captured in their territories and immediately placed in a dyadic contest paradigm that allowed for unrestricted contact between frogs. Males were paired opportunistically as soon as they were captured, for a total of 55 fighting trials, and later measured for body characteristics (within several hours of capture). To eliminate the possible influence of prior testing on a male's motivation to interact with conspecifics, no male was tested more than once.

Males were allowed to acclimate for 5 minutes under clear enclosures on opposite ends of a clear acrylic neutral arena (L=61cm, W=20cm, H=20cm) in the field. After 5 minutes, the acclimation enclosures were removed and the males were allowed to interact freely for 15 minutes. During the interaction period observers scored frequencies of common aggressive behaviors (Table 3.1; following Baugh and Forester 1994), as well as latency to initiate calling, which was used as a proxy for territorial motivation and aggressiveness.

Field Territoriality Tests

To test whether male brightness correlates with his response to a simulated agonistic threat in his territory, we conducted a series of behavioral observations of 109 calling territorial males in June-July of 2012. A small speaker (Altec Lansing iM-237) was placed on the ground at a distance of 60cm away from each male, measured horizontally from directly below the male's perch. As a visual stimulus, we placed a 3D-printed and hand-painted plastic model on top of the speaker (Fig 3.1; model from Turbosquid.com, printing by Shapeways, Inc). Following speaker placement, the male was observed for 5 minutes to allow him to habituate to the presence of the speaker and to collect data on his baseline activity.

A recording of a male call was then broadcasted using a SanDisk Sansa Clip+ MP3 player, and the male's responses to the call were recorded for 5 minutes. The acoustic stimulus consisted of a 15s long segment of an average male call alternating with 15s of silence. The recording of a Solarte male of average dorsal brightness and exhibiting average call characteristics (dominant frequency, call rate, call duration, duty cycle, pulse rate) out of a sample of 41 males recorded in 2009 was used as the stimulus call. The sound pressure level of the stimulus call playback was ~61 decibels (dB SPL re 20 mPa), as measured 60cm from the speaker in the field using a Pyle PSPL01 Mini digital sound level meter. During the playback, we focused on behaviors that could unambiguously be assigned as responses to the playback rather than nearby conspecifics, including the male's latency to orient to the speaker (male turns body to face in the direction of the speaker), whether the male approached the speaker (within a distance of 10cm or less), and whether the male interacted with the model frog (oriented body towards model and called while standing on the speaker, made contact with the model, or tackled the model).

Body Measurements

Males were taken to STRI following behavioral observation and measured on the day of capture. All males were measured for body length (snout-vent length, or SVL), mass (to the nearest 0.01g), spectral reflectance, body temperature at the dorsal surface (within 0.1C), and were photographed on a standard background against a ruler. Body temperature was measured using an infrared laser thermometer (Mastercool, Randolph NJ) immediately prior to spectral reflectance measurements for all frogs. In 2010, SVL was measured from

photographs using ImageJ software (Rasband 2012) and with manual calipers. SVL was measured using only digital calipers in 2012. In 2010, because photographs provided more accurate and precise body length estimates than those taken with calipers, we used the ImageJ measurements for these analyses. 11 frogs were not photographed using standard photograph conditions in 2010; thus, a dataset of 138 males from this population measured during that field season was used to impute missing ImageJ SVL values using k-nearest neighbor averaging of caliper/photographed SVL measurements using the R *imputation* package (Wong 2013).

Spectral reflectance measurements were taken at the head and dorsum (two measurements per region in 2010 and four in 2012) using an EPP2000 UV-VIS portable spectrometer and R600-8 UV-VIS-SR reflectance probe (StellarNet Inc., Tampa, FL) and a PX2 Xenon flash lamp outfitted with a custom-made 50Hz trigger input (Ocean Optics, Dunedin, FL). Spectralon white standard measurements were taken frequently to account for lamp drift. Dorsal reflectance spectra were obtained by averaging dorsal reflectance measurements of the head and dorsum. Averaged dorsal reflectance spectra were used to calculate the total reflectance flux (referred to in text as "brightness": $[\sum_{300nm}^{700nm} R(\lambda)]$), a perceptually unbiased estimate of male brightness.

Statistical Analysis

All statistics were performed in R 2.15.1 (R Development Core Team 2012). Correlations among predictor variables violate the assumptions underlying the statistical models described below. Brightness and SVL were weakly positively correlated in the 2010 dyadic contest dataset ($N = 110$; $t = 2.43$, $P = 0.017$; Multiple $R^2 = 0.05$); both traits were included in those models to isolate their independent effects. SVL and mass were positively correlated in the 2012 dataset ($N = 94$; $t = 6.158$, $P < 0.0001$, Multiple $R^2 = 0.29$), we therefore only used SVL and brightness as male traits in those models. Significance of model predictor variables was assessed using Wald and likelihood-ratio χ^2 statistics calculated from Type II analysis of deviance/variance tests in the *car* package (Fox and Weisberg 2011). Significance of overall models was assessed by comparisons to those fitted with only an

intercept term.

Staged Dyadic Contests

We tested two hypotheses concerning male brightness and aggression: *(1)* a male's brightness predicts his readiness to initiate territorial interactions, and *(2)* asymmetries in brightness between males predict the outcomes of dyadic contests. Calls are a central component of agonistic interactions between *O. pumilio* males (Baugh and Forester 1994; Pröhl 2005). Calling behavior also appears to be a reliable predictor of dominance outcomes in *O. pumilio* (Baugh and Forester 1994) and in *Eleutherodactylus coqui,* another small neotropical frog (Stewart and Rand 1991). Therefore, we assessed whether brighter males more readily initiated aggressive interactions in these trials by calling.

Of the 51 males that called, the latency for a male to start calling was modeled using a multivariable generalized estimating equation (GEE), using Poisson distribution with log link and an exchangeable correlation structure in the *geepack* package, and including male brightness, SVL, and mass as covariates (Højsgaard et al. 2006). This type of model produces estimates similar to those of generalized linear models, but with the estimated variances adjusted for the correlation of behavioral outcomes within each male pair, while also being permissive to violations of the distributional assumptions of mixed models (Zuur et al. 2009; Hardin and Hilbe 2012).

We next sought to determine whether trait asymmetries between paired males impacted the level of aggression/interaction exhibited in those trials, as a male's behavior will likely be influenced by the size of his rival's traits relative to his own (Enquist et al. 1990). Asymmetries in male traits were calculated by taking the absolute value of the logged ratio in trait values between the two males in a given trial (calculation is equivalent to that used in Enquist et al. 1990):

$$= |\log_{10}(\text{Value}_{\text{Male 1}} / \text{Value}_{\text{Male 2}})|$$

These asymmetries were calculated for brightness, SVL, and mass, and were included as covariates in the models described below.

Only 11 out of the 55 trials involved highly aggressive behaviors such as charges,

grapples, pinning of the other male, and chases, consistent with past studies of *O. pumilio* behavior (Pröhl and Berke 2001). We first used calling behavior to assess the level of aggression exhibited by the pair, coded as an ordinal response variable (no males called, one frog in the pair called, or both called), and modeled using ordinal logistic regression with the *polr* function in the *MASS* package (Venables and Ripley 2002). We also used Wilcoxon rank sum tests to determine whether trait asymmetries differed in escalated aggression trials where both males called (8 trials) versus less aggressive trials where only one or none of the frogs called.

In many taxa that have been studied, initiators are often the winners of agonistic interactions (e.g., Bekoff and Scott 1989; Jackson 1991; Stewart and Rand 1991; Hsu et al. 2009). We therefore performed a Chi-square goodness-of-fit test, testing the hypothesis that the initiator (first male to call) of an interaction was equally likely to rank above or below his rival in terms of brightness, SVL, or mass. Furthermore, in the majority of contests (35 out of 55) only one male was aggressive (=called). We therefore tested whether trait asymmetries influenced the likelihood that the brighter or duller male of the pair was the sole aggressor, using a binomial generalized linear model (GLM) with logit-link.

Finally, we assessed the influence of trait asymmetries on the likelihood of a trial resulting in the most frequently documented highly aggressive behavior (charges: 9/55 trials) using a binomial GLM. We explored this question both by calculating the trait asymmetries as described above, and by classifying males into brightness categories (brighter than the mean for the dataset; duller than the mean). In the second case, we predicted that males classified in the same brightness category (both brighter than average, or both duller than average) might be more likely to escalate and show highly aggressive behaviors than males falling into disparate categories.

Field Territoriality Tests

By testing male aggressive response to a simulated territorial intrusion in naturalistic settings, we tested two additional hypotheses regarding male brightness: *(1)* brighter males more readily respond to an intruder in their territory, and *(2)* brighter males are more likely to be interacting with conspecifics (as predicted by Maan and Cummings 2009; Crothers et

al. 2011). 15 of the 109 males observed in the playback experiments had been previously captured earlier in the field season (as identified by toe clippings found on frogs after behavior observations), and were removed from the analysis to ensure no accidental retesting. 20 males were calling to nearby conspecifics (<60cm away) during their baseline observation period; this was coded as a dichotomous variable and included in the analysis to account for any effect of a conspecific's presence on male response to the playback.

We first investigated whether a male's body characteristics were correlated with the probability that he was associating with a conspecific by fitting a binomial GLM with male brightness and SVL as covariates. Next, the latency for a male to orient to the model (for the 67 males that oriented) was fitted with a Poisson GLM including brightness and SVL as covariates, and correcting standard errors for overdispersion using a quasi-model where the variance is given by $\phi*\mu$, where μ is the mean and ϕ is the dispersion parameter. Whether a male approached the speaker was coded as a dichotomous variable and modeled using a binomial GLM with brightness, SVL, initial perch height, and whether a male was associating with a conspecific during the baseline included as covariates. Finally, very few males (N=9) climbed onto the speaker and interacted with the model frog, thus we used an exact binomial test to see whether a greater number of males exceeding the average brightness or SVL attacked the model than expected by chance.

3.3 RESULTS

Staged Dyadic Contests

Effects of Male Brightness on Behavior

Brighter males called significantly faster than duller males (Fig 3.2a; N = 51; Poisson GEE; overall model P = 0.007; $Wald_{Brightness} = 4.02$, $P_{Brightness} = 0.045$). Male SVL and mass had no effect on call latency (Fig 3.2b,c; Wald = 2.00_{SVL}, 0.00_{Mass}, P = 0.157_{SVL}, 0.995_{Mass}).

Effects of Brightness Asymmetries Between Males on Behavior

Interactions often involved only a handful of calls (median = 4 calls). The magnitude of the asymmetry in brightness, SVL, and mass had no impact on whether none, one or both frogs called in a given pair (N = 55; ordinal logistic regression; overall model P = 0.470; χ^2 = $1.40_{Brightness}$, 1.04_{SVL}, 0.34_{Mass}, All Ps > 0.24). However, the brightness asymmetry between males was significantly smaller in the 8 trials where both males called versus less aggressive trials where only one or none of the males called (Fig 3.3a; Wilcoxon rank sum test: W = 103, P = 0.042), while there was no significant effect of SVL or mass asymmetry on these outcomes (Fig 3.3b,c; Wilcoxon rank sum tests: *SVL*: W = 198, P = 0.821, *Mass*: W = 235, P = 0.267). Furthermore, males that initiated interactions within a pair were not equally distributed among the brightness, SVL, and mass categories (N = 43; χ^2 contingency table test, χ^2 = 16, df = 7, P = 0.025). Initiator males were more often the brighter, heavier, and longer of the pair than expected by chance (Table 3.2). When the analysis was limited to the 27 trials where males were matched for body length (difference < 1 SD), the brighter male of the pair was significantly more likely to initiate than the duller male (N = 14 trials with brighter initiator, 5 with duller; Chi-square goodness-of-fit test: χ^2 = 4.26, df = 1, P = 0.039).

In the 35 trials where there was only one aggressor (=only one male called), the magnitude of the brightness asymmetry between the frogs had a significant positive impact on the probability of the brighter male of the pair being the aggressor, while SVL asymmetry had a significant negative impact (Fig 3.4; Binomial GLM; overall model P = 0.035; Wald = $4.07_{Brightness}$, 3.97_{SVL}, 1.60_{Mass}, P = $0.044_{Brightness}$, 0.046_{SVL}, 0.206_{Mass}). Finally, trait asymmetries did not have an effect on the probability of males charging one another in a trial (N = 55; binomial GLM: Wald = $2.086_{Brightness}$, 0.070_{SVL}, 0.045_{Mass}, P = $0.15_{Brightness}$, 0.79_{SVL}, 0.83_{Mass}). However, charges were more likely to occur in trials where males were both ranked in the same brightness category than in trials where males were mismatched in brightness rank (binomial GLM; Wald = 4.81; P = 0.028).

Field Territoriality Tests

There was no relationship between a male's brightness or SVL and whether he was interacting with a conspecific before the playback test (Binomial GLM: N = 94, Wald =

$0.207_{Brightness}$, 0.447_{SVL}, P = $0.65_{Brightness}$, 0.50_{SVL}). Brighter males oriented to the speaker significantly faster than duller males, and there was no significant effect of SVL; however the overall model was only significant when brightness was included as the single covariate (Fig 3.5; N = 67; "quasi-Poisson" GLM: F = 5.39, P = 0.023). There was a marginally significant positive effect of male perch height on the probability of a male approaching the speaker (N = 94; Binomial GLM, Wald = 3.72, P = 0.054), but no effect of brightness, SVL or presence of a conspecific on this behavior (all Wald < 0.81, all Ps > 0.370). Finally, 6 out of the 9 males that interacted with the model frog were brighter than average males, and 7 were longer than average males; these results did not significantly differ from chance (exact binomial tests: $P_{Brightness}$= 0.508; P_{SVL} = 0.180).

3.4 DISCUSSION

Aggressive interactions are often settled through weapons (Emlen 2008) or comparisons of phenotypic traits that provide information on asymmetries in body size (Maynard Smith 1974; Davies and Halliday 1978; Huntingford and Turner 1987) or motivational state (e.g., Enquist and Leimar 1987; Kotiaho et al. 1999; Hofmann and Schildberger 2001). In this aposematic frog species, the brightness of a male's conspicuous orange-colored dorsum was a more reliable indicator of a male's willingness to initiate aggressive interactions than standard phenotypic characters of male size (SVL or mass). Assessment signals can serve to counter the costs of agonistic interactions by settling the interactions prior to physical contact. Studies across taxa including red deer (Clutton-Brock and Albon 1979), cichlids (Enquist et al. 1990), field crickets (Hofmann and Schildberger 2001) and wolf spiders (Kotiaho et al. 1999) show that the majority of territorial disputes are settled via non-contact phenotypic comparisons, while fewer disputes are settled via contact interactions. We observed a similar pattern here with the Solarte population of strawberry poison frogs, where out of 55 dyadic encounters only 11 involved some form of physical combat (grappling, charges, etc.). This pattern suggests that males are using phenotypic

assessment to regulate agonistic escalation, and dorsal brightness appears to be the most reliable cue of a male's aggressive intent in this species.

Here, we used natural variation in male traits to assess the influence of male brightness (and other body attributes) on male aggression and the outcomes of dyadic male contests. We find that brighter males are more aggressive, calling faster than their duller counterparts when confronted with a conspecific rival (Fig 3.2) and more readily attending to male calls within/near their territories (Fig 3.5); these results agree with and expand upon past laboratory findings that males pay attention to rival brightness when placed in agonistic dichotomous choice trials that manipulate male perception of rival brightness (Crothers et al. 2011). Males that initiated aggression in the dyadic contests (=first callers) were significantly more likely to be both the brighter and longer male of the two in a contest (Table 3.2), though brighter males were also more often the aggressor of the pair than expected by chance in trials where males were approximately matched for body length. Furthermore, in the >60% of trials where only one male called, the magnitude of the brightness asymmetry between the males (holding the effect of asymmetry in body size constant) predicted the odds of the aggressor being the brighter male (Fig 3.4a). We also provide evidence that brightness asymmetries between males in dyadic contests can be used to settle interactions without the need for further escalation (Fig 3.3a; Fig 3.4a), as predicted by status signal hypotheses (Rohwer 1975; Enquist et al. 1990; Maynard Smith and Harper 2003). Finally, our results indicate that brighter males were more likely to be the aggressor in these trials when SVL asymmetries were small (Fig 3.4b), implying that brightness information may be relied upon when body size asymmetries are difficult to perceive. Thus, this study, combined with past research (Crothers et al. 2011), shows that males respond to the brightness of rivals when controlling for other factors (such as body size and behavior), and that male brightness predicts his aggression and thus his likelihood of dominating other males.

Signal brightness can function as a reliable indicator of an individual's health, aggression, and/or foraging ability across a broad range of taxonomic groups (e.g., Hamilton and Zuk 1982; Folstad and Karter 1992; Ryan and Keddy-Hector 1992; Andersson 1994). Furthermore, warning signal brightness appears to co-vary with some fitness-related traits in this species (advertisement call characteristics and body temperature; Crothers et al. 2011). If

male warning signal brightness is functioning as a classic indicator signal used in aggressive assessment in *O. pumilio*, we would predict that contests with larger asymmetries in brightness should be less aggressive and contests with small asymmetries in brightness should reach a high state of aggressiveness (Enquist et al. 1990; Maynard Smith and Harper 2003). In our study, the level of brightness asymmetry did not correlate with whether males charged one another in a trial (N = 9 trials). However, we found some evidence that supported a role of brightness as an agonistic signaling trait, in that brightness asymmetries between males were smaller in escalated aggression trials (where both competitors called; Fig 3.3a), and brighter males were more likely to be the sole aggressor in trials with large brightness asymmetries (Fig 3.4a). Thus, our results indicate that warning signal brightness correlates with aggressiveness and can be used as a cue during aggressive interactions, and provide some evidence that its pattern of influence on these interactions is consistent with an agonistic assessment mechanism.

Our body measurements were taken within several hours of the dyadic behavior tests, so we cannot entirely exclude the possibility that the outcomes of our tests impacted the brightness of the males. However, very few of these interactions involved energetically costly behaviors such as protracted calling bouts or bodily contact. Amphibians are ectothermic, so changes in an animal's body temperature can provide some information about the metabolic costs of these trials. For a subset of trials we measured male body temperature both before the trial began and immediately afterwards (N = 52 males). When we compared the change in body temperature between three types of frogs (*(a)* initiators, *(b)* non-initiators, and *(c)* frogs in trials where no males called), we found no difference between the three groups (Kruskal-Wallis rank sum test; $\chi^2 = 1.83$, df = 2, P = 0.401). Furthermore, the brightness of males in trials where males were highly aggressive and charged at one another (N = 9 trials) did not differ from the brightness of males in trials involving no charges (N = 46 trials; Wilcoxon rank sum test; W = 922, P = 0.45).

Our findings indicate that brighter males may be greater territorial threats than duller males, and because they are also likely to be more noticeable by virtue of their enhanced conspicuousness may be more readily approached by male competitors (as found by

Crothers et al. 2011). Preferential aggression towards brighter males has been observed in lazuli buntings, where dull young males are able to settle in high quality territories because they are largely ignored by brighter, older males (Greene et al. 2000). Though past laboratory studies revealed a widespread female preference for brighter males in this population of *O. pumilio* (Maan and Cummings 2009), we did not find that brighter males were more likely than duller males to have conspecifics in their territories. More comprehensive examination of male mating success in the wild is necessary to determine if brighter, more aggressive males enjoy higher reproductive success.

It is growing increasingly evident that the forces of selection are all impacting a single, quantifiable trait in poison frogs: warning coloration. Here, we add another piece to the puzzle: the apparent co-option of warning coloration as a male status signal. We find that warning signal brightness in the exceptionally conspicuous Solarte population functions as an agonistic status signal, correlating with male behavior and predicting the outcomes of male-male interactions. These results indicate that brighter males may be superior competitors, more readily obtaining and maintaining their territories. Though indicator models of sexual selection have generally not been considered as a mechanism that drove color diversification across the populations of the archipelago, this form of sexual selection may be operating within populations to drive and maintain aposematic signal variation, the raw material for selection to act upon. In conclusion, our finding that a warning signal functions as a status signal speaks to the evolutionary lability of aposematic traits and their utility in investigating general patterns of signal evolution.

3.5 TABLES

Behavior	Description
Call	Male orients body towards other frog and inflates vocal pouch while producing a rapid burst of chirps.
Track	Male orients body towards other frog without moving forward.
Approach	Male orients body towards other frog while moving forward.
Move away	Male orients body away from other frog while moving.
Charge	Male runs towards the other frog and veers away without making physical contact or makes physical contact with the other frog.
Grapple	Male seizes other frog's limbs.
Pin	Male sits on other frog or wraps body around the dorsal surface of other frog.
Chase	Male pursues other frog while his opponent retreats.
Escape	In response to aggression from other frog, male rapidly moves away from aggressor towards a distant side of the test chamber.
Statue	In response to aggressive behavior from other male, the submissive frog remains frozen in one position.

Table 3.1: Behaviors scored during staged dyadic contests. Behaviors are a modified version of those described in Baugh and Forester (1994).

		Heavier	Lighter
Brighter	**Longer**	10*	8
	Shorter	3	5
Duller	**Longer**	6	1§
	Shorter	1§	9

Table 3.2: Frequencies of trials where the initiator (first caller) was greater than or less than his competitor in terms of brightness, body length (SVL), and mass. Symbols indicate when the frequency violated the equal probability assumptions of the 3-way contingency test (*=event occurred more often than expected; §= event occurred less often than expected).

3.6 FIGURES

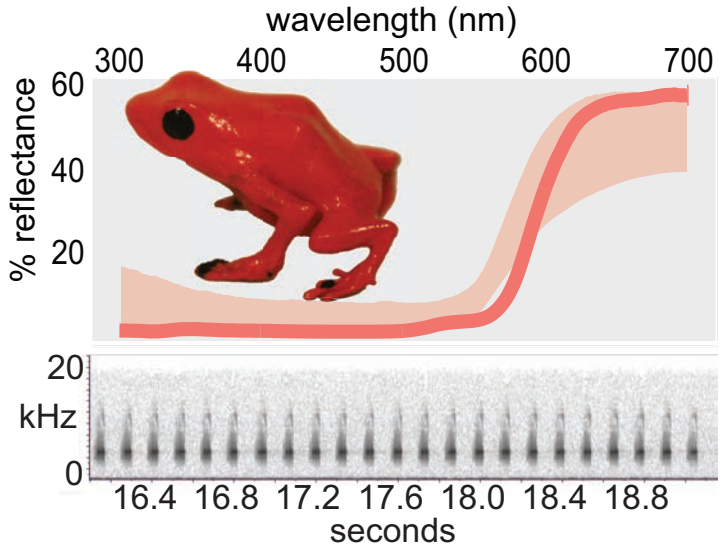

Figure 3.1: Photograph and spectral plot of plastic model and spectrogram of acoustic stimulus used in playback experiments. The red line on the spectral plot depicts the reflectance of the plastic stimulus model across the measured wavelengths, and is bounded by the reflectance of the dullest and brightest males of a sample of 139 males measured in 2012 (pale tan area).

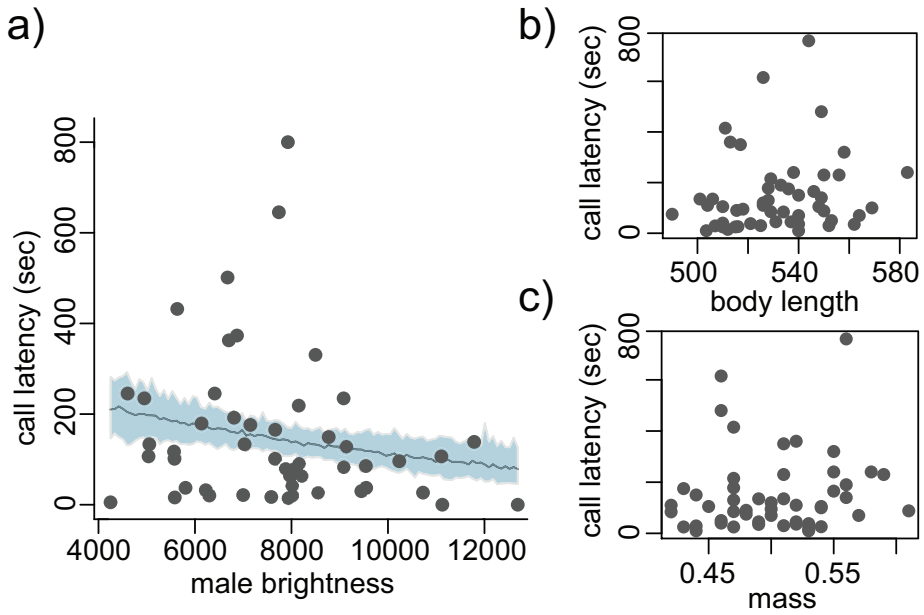

Figure 3.2: Relationship between males' latency to call and body characteristics. (A) male brightness ($\sum_{300nm}^{700nm} R(\lambda)$]), (B) body length (in arbitrary units), and (C) mass (g). Dots represent data points, and the black line and shaded area represent the predicted line and 95% confidence intervals of the GEE model, respectively.

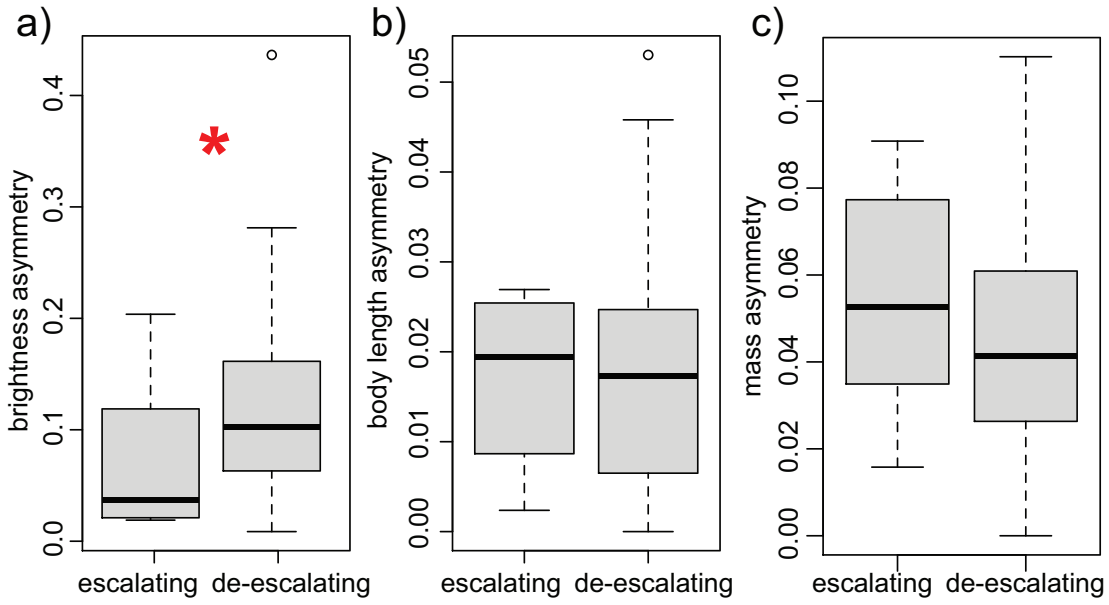

Figure 3.3: Brightness asymmetries by whether trials were escalating or de-escalating. Box-and-whisker plots of (A) brightness asymmetry, (B) body length asymmetry, and (C) mass asymmetry between males in a given trial according to whether the trial was escalating (both males called in the trial) or de-escalating (only one male called or no males called). Boxes span the first and third quartile of the data, and horizontal black lines represent the median. Whiskers span the range of the data, excepting outliers (open circles).

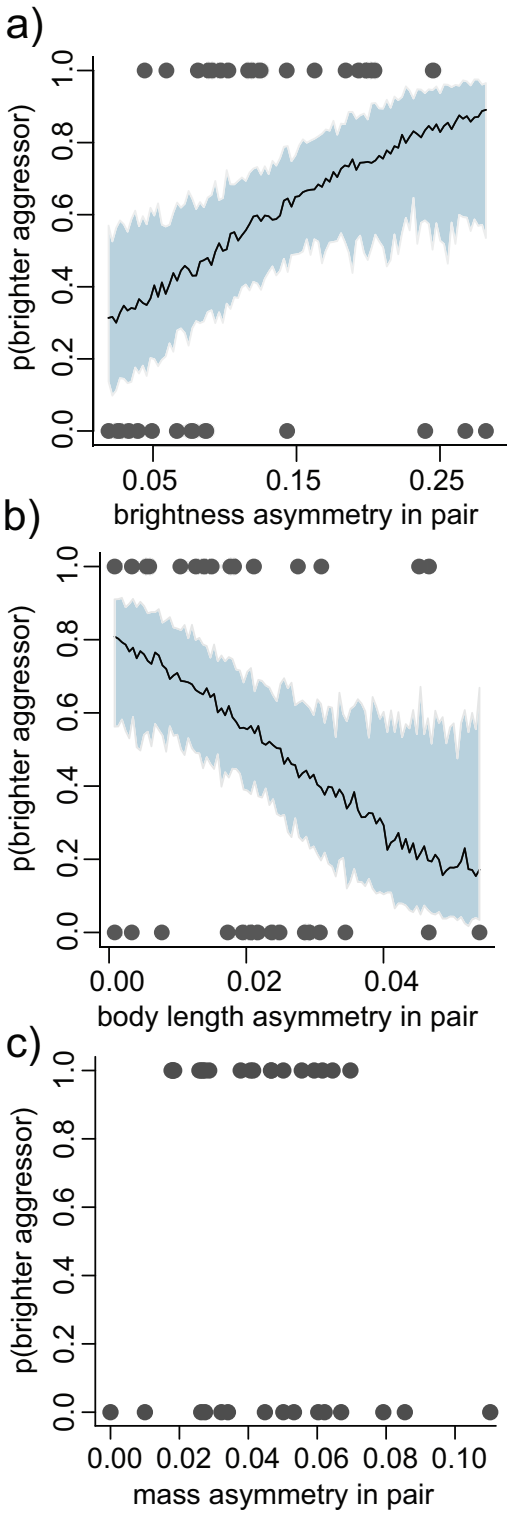

Figure 3.4: Probability of brighter male being the aggressor according to body characteristic asymmetries. Probability of the brighter male of a pairing being the aggressor (in trials where only one male called) as a function of (A) the brightness asymmetry between the males, (B) the body length asymmetry between the males, and (C) the mass asymmetry between the males. Dots represent data points, and the black line and shaded area represent the predicted line and 95% confidence intervals of the binomial GLM model, respectively.

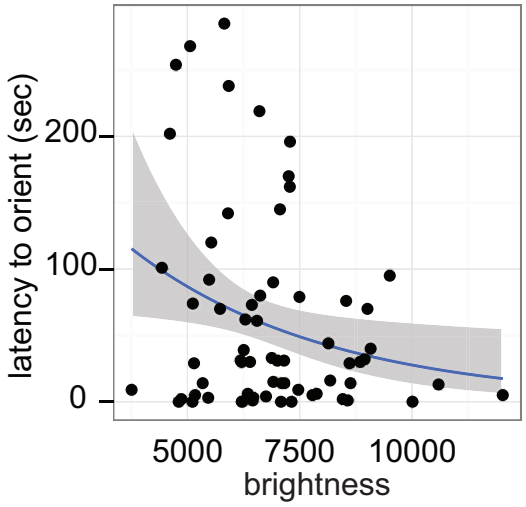

Figure 3.5: Relationship between male brightness and latency to orient to speaker (turning body to face speaker) during simulated territorial challenge trials. Line and shaded area flanking the line represent the predicted line and smoothed 95% confidence intervals of the "quasi-Poisson" GLM, respectively, while dots represent data points.

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CHAPTER 4:

Warning signal brightness co-varies with toxicity but not classic measures of body condition in an exceptionally conspicuous poison frog population

ABSTRACT

Variation in aposematic ("warning") patterns is found throughout the animal kingdom, seemingly conflicting with classic theories of signal evolution. Because aposematic species are highly conspicuous, we may expect that their warning signals will be repeatedly co-opted for use in conspecific communication systems, and such a phenomenon has been demonstrated in several aposematic species. In the strikingly bright and toxic Solarte population of the strawberry poison frog (*Oophaga pumilio)*, bright male warning coloration has been co-opted as a sexual signal. Females of this population prefer brighter males and male brightness evidently functions as an agonistic indicator trait. Here, we investigate which physiological characteristics correlate with bright male warning coloration to gain insights into the evolutionary stability of this trait and what mechanisms help to maintain the brightness variation observed within this population. We find that although male brightness does not correlate with two classic correlates of sexually selected traits (circulating testosterone and total carotenoids in the skin), it does show a negative relationship with aggregate pumiliotoxin in the skin, which is considered the major class of defensive alkaloids in this species. Because the alkaloids used in chemical defense in this species are acquired from dietary sources, the magnitude of a male's warning signal brightness can potentially provide viewers with reliable information regarding foraging ability, territory quality, health, and/or genetic quality.

4.1 INTRODUCTION

Colorful animal signals often represent elegant demonstrations of the evolutionary outcomes of sexual selection (Darwin 1871). But some colorful signals, known as "aposematic" signals, have evolved through natural selection to advertise to predators that a defended prey item is unprofitable to attack (Wallace 1867). A classic hypothesis predicts that these traits should converge on a standard form, reducing the number of noxiously defended signals for predators to learn and lowering the number of sacrificial individuals necessary to 'train' predators (Müller 1879). However, variation in aposematic patterns has been extensively demonstrated (e.g., Siddiqi et al. 2004; Bezzerides et al. 2007; Nokelainen et al. 2011; Willink et al. 2013), seemingly conflicting with theory. Because aposematic species are highly conspicuous, we may expect that their warning signals will repeatedly be co-opted for use in conspecific communication systems; this pattern has been found in several aposematic species (Jiggins et al. 2001; Maan and Cummings 2008; Nokelainen et al. 2011).

Evidence for multifarious selection on warning coloration is rapidly accumulating for one of the most variably colored aposematic species: the strawberry poison frog (*Oophaga [Dendrobates] pumilio*). Insular and coastal populations of this species in the Bocas del Toro archipelago of western Panama come in >15 color patterns (Daly and Myers 1967; Siddiqi et al. 2004), and in some populations considerable intra-population variation in color and brightness have been documented (Richards-Zawacki and Cummings 2011; Crothers and Cummings 2013; L.R. Crothers, *unpublished data*). Though there is a positive correlation between population brightness and average toxicity *across* phenotypically distinct archipelago populations (Maan and Cummings 2012), brightness variation *within* populations appears to be imperceptible to the putative major predator of the species (birds: Crothers and Cummings 2013).

Sexual selection has been favored as a major force driving the enhanced warning signal brightness of the derived insular populations (Summers et al. 1997; Maan and Cummings 2009; reviewed in Cummings and Crothers 2013) where females show a widespread preference for brighter males (Maan and Cummings 2009) and bright warning coloration appears to have been co-opted as an agonistic status signal within the bright

orange and highly toxic Solarte population (Crothers et al. 2011; Crothers and Cummings, *in review*). Furthermore, we observe substantial variation in brightness and long-wave chroma (~redness) in this population (Crothers and Cummings 2013).

Theory provides several possible avenues for the maintenance of signal diversity in the presence of directional sexual selection (Kirkpatrick and Ryan 1991; Rowe and Houle 1996). Many sexually selected traits are intrinsically linked to aspects of an individual's physiological phenotype (Andersson 1994). Signal brightness, for example, has been shown to function as a reliable indicator of an individual's health, aggression, and foraging ability across a broad range of taxonomic groups (e.g., Hamilton and Zuk 1982; Folstad and Karter 1992; Ryan and Keddy-Hector 1992; Andersson 1994). The simultaneous use of *O. pumilio*'s bright warning coloration in intraspecific and aposematic communication implies that this signal may show a predictable relationship with some aspect of physiology and body condition, though how these relationships play out at the within-population level are not yet known.

Warning coloration may be a condition-dependent trait in many populations of this species, as it can correlate with body temperature and call characteristics (Crothers et al. 2011) and appears to fluctuate according to diet and across time (J. Yeager, *pers. comm.*, L.R. Crothers, *unpublished data*). There are several candidate biochemicals that may co-vary with warning color or brightness. First, the development of sexually selected bright signals is often testosterone-dependent (Folstad and Karter 1992; Johnstone and Norris 1993; Sinervo et al. 2000; reviewed in Whiting et al. 2003). Second, color pigments acquired from dietary sources are predominant in bright orange/red signals (Kodric-Brown 1989; McGraw 2005), and represent candidate molecules for maintaining signal honesty in both sexual and aposematic signals (Andersson 1994; Olson and Owens 1998; McGraw and Ardia 2003; Bezzerides et al. 2007; Blount et al. 2009; Hill and Johnson 2012). Supplementation of multivitamins also appears to rescue the natural color phenotype of captive *O. pumilio* of some populations (J. Yeager and C. Richards-Zawacki, *pers. comm.*). Finally, brightness or coloration may co-vary with skin toxins within a population (Blount et al. 2009; Lee et al. 2011; Holen and Svennungsen 2012), as they do across populations of this and other aposematic species (Bezzerides et al. 2007; Maan and Cummings 2012). Here, we investigate

several potential physiological correlates of bright warning coloration in the phenotypically variable and sexually dimorphic *O. pumilio* Solarte population, to determine whether circulating testosterone, skin pigments, or dietarily derived skin toxins correlate with male brightness or coloration.

4.2 METHODS

Body Measurements

Calling territorial adult males were located in the field during early daytime hours in August-September of 2011 and July of 2012 on Isla Solarte, in Bocas del Toro, Panama (N 09^{O}20.014' W 82^{O}13.197'). Males were captured and kept individually in plastic 475mL deli containers moistened with ultraviolet (UV) purified water until body measurements were taken. In 2011, males were measured in a tent in the field within several hours of capture. In 2012, males were first transported to the Smithsonian Tropical Research Institute (STRI) in Bocas del Toro, Panama and measured there within 24 hours of capture. All males were measured for body length (snout-vent length, or SVL), mass (to the nearest 0.01g), spectral reflectance, and body temperature at the dorsal surface (within 0.1C), and were photographed on a standard background against a ruler, as done previously (Crothers et al. 2011; Crothers and Cummings, *in review*). In 2011, SVL was measured from photographs using ImageJ software (in arbitrary units; Rasband 2012). SVL was measured using digital calipers in 2012 (to the nearest 0.1 mm).

Spectral reflectance measurements were taken at the head and dorsum (two measurements per region in 2011; four measurements per region in 2012) using an EPP2000 UV-VIS portable spectrometer and R600-8 UV-VIS-SR reflectance probe (StellarNet Inc., Tampa, FL) and a PX2 Xenon flash lamp outfitted with a custom-made 50Hz trigger input (Ocean Optics, Dunedin, FL). Spectralon white standard measurements were taken frequently to account for lamp drift. Males captured in 2011 were briefly housed individually at STRI until they were returned to their territories in the field.

Analysis of Brightness

Dorsal reflectance spectra were obtained by averaging measurements of the head and dorsum (N=4 for 2011 and N=8 for 2012). Averaged dorsal reflectance spectra were used to calculate both the total reflectance flux [$\sum_{300nm}^{700nm} R(\lambda)$]), a perceptually unbiased estimate of male brightness, and color (long-wave chroma). Long-wave chroma assesses the proportion of the reflectance flux in the long-wave band: $\left[\frac{\sum_{i=600nm}^{700nm} R(\lambda)}{\sum_{i=300nm}^{700nm} R(\lambda)}\right]$.

Circulating Testosterone

Twenty-three calling males were collected in the field in 2011, had their body measurements (described above) taken on the day of capture, and were allowed to habituate in individual terrariums at STRI (~37cm x 22cm x 24cm) containing water, leaf litter, and arthropods for at least 24 hours prior to blood collection. Blood was collected from the orbital sinus using a heparinized capillary tube with a tapered end produced with a micropipette puller. One frog died following blood collection. The others were allowed to recover for at least 24 hours at STRI before being returned to their points of capture in the field. Blood was also collected from 6 adult males from a colony maintained in the Richards-Zawacki laboratory at Tulane University in July of 2013 in order to perform a serial dilution validation for the hormone analysis. Immediately following sacrifice of these frogs via double pithing, blood was collected from an incision in the right hind leg with a heparinized micropippetor tip. All blood samples were immediately centrifuged at ~10,000 rpm for 6 minutes and the plasma layer collected and frozen for several days on dry ice while in transport to the University of Texas, where they were maintained at -20C until hormone analyses were performed in February of 2014.

We measured circulating levels of total testosterone using ELISA (Enzo Life Sciences, Cat # ADI-900-065). *O. pumilio* plasma samples were analyzed for parallelism with the kit's standard curve using a series of 6 dilutions from the pooled plasma stock (1:10, 1:20, 1:40, 1:80, 1:160, and 1:320). The dilutions ran parallel to the standard curve

(homogeneity of slopes ANCOVA: $F_{1,7}$=0.019, P=0.895), validating the kit use with this species. Individual male plasma samples were diluted at 1:64 (16 samples), 1:86 (5 samples), 1:142.5 (1 sample), and 1:213.5 (1 sample) in assay buffer and the kit protocol was strictly followed. The lower dilution concentrations used for some samples were due to exceptionally small available plasma quantities (<2μL) for those samples. All samples were run in duplicate. The plates were read with a conventional plate reader at 405 nm (SpectraMax M3, Molecular Devices). To compute circulating testosterone levels, the percent bound for each of the standards was calculated and a logarithmic curve was generated. This curve was then used to compute the circulating testosterone levels for the males (in ng/mL). Intra-assay variation was 17.6%, and inter-assay variation was 7.14%.

Skin Carotenoids

For a subset of the males caught in 2012 (N=10) we recorded male perch height in his territory prior to capture. Immediately following body measurements at STRI (size and spectral measurements), all 20 males were sacrificed by double pithing. A small sample of dorsal skin was collected for skin alkaloid analysis from each male, as described below. The remaining dorsal skin was dissected and frozen in liquid nitrogen for transport to the United States, after which samples were stored at -80C until their carotenoid content was analyzed in October of 2013. Carotenoid levels in the dorsal skin tissue were quantified (in μg of carotenoid per g of tissue) using high performance liquid chromatography (HPLC) in the laboratory of Dr. Kevin McGraw at Arizona State University, following a modified version of previously established protocols (McGraw et al. 2006).

In brief, carotenoids were extracted using a micronizer in the presence of solvent (1.4mL hexane:tert butyl methyl ether, 1:1, v/v), using 0.1g of skin. Tissue and solvent were centrifuged, and the supernatant was recovered and dried down for carotenoid analysis. HPLC analyses follow those in McGraw et al. (2006), using a Waters 2695 instrument (Waters, Milford, MA). Because of the presence of ketocarotenoids in the samples, the analytical method was slightly modified. First, the HPLC column (Waters YMC Carotenoid column, 5mm, 4.6mm #250mm) was pretreated with 1% orthophosphoric acid in methanol

for 30 min at 1mL/min. Secondly, solvent composition and flow rate were altered to optimize separation of different ketocarotenoids. At a constant flow rate of 1.2mL/min, an isocratic elution with 42:42:16 (v/v/v) methanol:acetonitrile:dichloromethane was first used for 11 min followed by a linear gradient up to 42:23:35 (v/v/v) methanol:acetonitrile:dichloromethane through 21 min, holding those conditions until minute 25 and finishing with a return to the original isocratic conditions from 25–29.5 min. Carotenoid types were identified by comparison to authentic standards from CaroteNature (Ostermundigen, Switzerland). External standard curves were used to quantify concentrations of each carotenoid type. One sample was lost during HPLC analysis, resulting in a final sample size of 19 males for this dataset.

Skin Alkaloids

A sample of mid-dorsal skin tissue was removed from the 20 recently sacrificed males described above using a 4mm diameter circular biopsy punch. Biopsies were consistently performed on the same side and approximate location on the dorsal surface. Tissue samples were stored individually at room temperature in methanol-filled glass vials for subsequent alkaloid analysis. Individual alkaloid fractions were prepared in April of 2013 from methanol extracts of each skin and characterized using gas chromatography in combination with mass spectrometry (GC-MS) using the methods detailed in Saporito et al. (2010), with final dilution volumes modified to accommodate the smaller tissue sizes. Comparisons of mass spectrometry properties and GC retention times with those described in previous studies allowed us to identify individual alkaloids for each skin (Daly et al. 1994; Saporito et al. 2010). All alkaloids within a fraction were quantified (in μg) by comparison of the alkaloid's peak area to the peak area of a nicotine internal standard.

Statistical Analysis

All statistical tests were performed in R 2.15.1 (R Development Core Team 2012). We first assessed the relationship between circulating testosterone (in ng/mL) and male

warning coloration by fitting two linear regressions (LM): one with male brightness and the other with long-wave chroma as predictor variables. Two samples were off the standard curve and were not included in these analyses, resulting in a final sample size of 21 males for the hormone analyses.

For the 10 males on which we collected perch data, we used Kendall's rank correlation to assess whether male brightness co-varied with a male's perch height (in m) in his territory in the field. We also tested the relationship between total skin carotenoids (μg/g) and male coloration by fitting two linear regressions: one with male brightness and the other with long-wave chroma as predictor variables. We also used linear regression to determine whether the proportions of the most abundant carotenoids (beta-carotene and xanthophylls) co-varied with the most abundant alkaloids (tricyclics and pumiliotoxins) within samples, because alkaloid sequestration, modification, and storage are believed to be oxidatively stressful for chemically defended organisms (Ahmad 1992; Blount et al. 2009).

Because the alkaloid dataset contained two exceptionally toxic males (>6x that of the median total alkaloids for the dataset), we converted total alkaloids in the skin (in μg), and total alkaloid diversity (number of unique alkaloids) to rank data. Small sample size (N=20) precluded the use of ordinal logistic regression, thus we modeled the relationship between male color measurements (brightness and long-wave chroma) and these alkaloid measures using Kendall's rank correlations. We also modeled the relationship between coloration and alkaloids/carotenoids with Wilcoxon rank sum tests using a dichotomous brightness and long-wave chroma measure (brighter or redder than the median versus duller or less red than the median total reflectance flux or long-wave chroma for the dataset, respectively; as in Crothers and Cummings 2013). Finally, we sought to determine whether brightness or long-wave chroma co-varied with total aggregate pumiliotoxins, considered the major class of toxic alkaloids found in the skin of poison frogs of the *Dendrobates/Oophaga* genera (Daly and Myers 1967; Daly et al. 1999).

4.3 RESULTS

Circulating testosterone levels ranged from 0.67 - 2.68 ng/mL (mean = 1.34, SD = 0.65). There was no relationship between circulating testosterone and male brightness (Fig 4.1a; LM: N = 21, t = 0.545, P = 0.592) or long-wave chroma (LM: t = -1.011, P = 0.325).

For the males with perch data, we found a significant positive relationship between male brightness and perch height (Fig 4.2; N = 10, Kendall's rank correlation: T = 36, P = 0.017). We identified 17 unique carotenoids within the samples analyzed (Table 4.1), and total carotenoid quantities ranged from 86.04 - 1421.51 μg/g. There was no relationship between male brightness/long-wave chroma and total dorsal skin carotenoids, both when assessed as a linear relationship (see Fig 4.1b for brightness plot; LM: N = 19, both $t < 0.94$, both $P > 0.360$) and when these color characteristics were coded as dichotomous variables (Wilcoxon rank sum test: *brightness*: W = 39, P = 0.661; *long-wave chroma*: W = 46, P = 0.968).

The proportion of tricyclic alkaloids was positively correlated with the proportion of beta-carotene (Fig 4.3a; LM: N = 19, t = 2.426, P = 0.027) and negatively correlated with the proportion of xanthophylls (Fig 4.3b; LM: N = 19, t = -2.548, P = 0.021). The proportion of pumiliotoxin alkaloids was negatively correlated with the proportion of beta-carotene (Fig 4.3c; LM: N = 19, t = -2.609, P = 0.018), but not correlated with the proportion of xanthophylls (Fig 4.3d; LM: N = 19, t = 1.639, P = 0.119).

There was a large range of both alkaloid quantity (1.59 - 153.41μg, median = 5.64, SD = 33.60) and alkaloid diversity (8 - 48 unique alkaloids, median = 13.5, SD = 9.38) within the small 4mm samples of dorsal skin (Table 4.2). Total alkaloids and alkaloid diversity were positively correlated (Kendall's rank correlation: N = 20, z = 3.375, P = 0.0007). There was no correlation between male brightness and total alkaloids (Fig 4.4a; Kendall's rank correlation: N = 20, z = -1.3627, P = 0.173) or alkaloid diversity (Fig 4.4a; z = -1.212, P = 0.225), or long-wave chroma and these measures (Kendall's rank correlations: both $P > 0.299$). However, when males were categorized into dichotomous brightness categories, brighter than average males had significantly less alkaloid diversity than duller than average males (Fig 4.4a; Wilcoxon rank sum test: W = 23, P = 0.043), but did not differ from duller males in terms of total alkaloids (Fig 4.4a; Wilcoxon rank sum test: W = 25, P =

0.063). Finally, there was a negative relationship between male brightness and total pumiliotoxin (Fig 4.4b; Kendall's rank correlation: N = 20, z = -2.160, P = 0.031), but no relationship between long-wave chroma and total pumiliotoxin (Kendall's rank correlation: z = 0.589, P = 0.556).

4.4 DISCUSSION

Bright ornamentation often functions as an indicator of body condition or territorial status in animals (reviewed in Andersson 1994; Whiting et al. 2003), and the condition-dependence of these traits can explain both their evolutionary stability (Maynard Smith and Harper 2003) and the extensive phenotypic variation that is often observed in conspicuous traits (e.g., von Schantz et al. 1999). Though the aposematic signal diversification of *O. pumilio* appears to result from a complex interplay between natural and sexual selection, sexual selection appears to be the major force promoting signal evolution within the highly conspicuous and toxic Solarte population and across insular populations of the archipelago (reviewed in Cummings and Crothers 2013; Gehara et al. 2013). Here, we sought to address the relationships between male warning signal brightness and several common physiological correlates of conspicuous sexual and aposematic signals. We find that though *O. pumilio* male warning signal brightness does not correlate with two classic measures of male body condition (total skin carotenoids or circulating testosterone levels), it does appear to negatively co-vary with a male's level of chemical defense.

Carotenoids are a candidate molecule for condition-dependence in many species, because they are derived dietarily in animals and can function as precursors in important redox homeostatic reactions (Olson and Owens 1998; Hill and Johnson 2012). Although the skins of *O. pumilio* contain a strikingly complex mixture of carotenoids (Table 4.1; K. McGraw, *pers. comm.*), skin characteristics such as brightness and long-wave chroma did not correlate with the quantities of these pigments in the skin. Though these results were unexpected, a lack of correlation between plumage coloration and carotenoid content has been observed in some birds (Saks et al. 2003), and pigments other than carotenoids can

often contribute to orange and red coloration in animals (e.g., pteridines; Bagnara 2003; McGraw 2005; Weiss et al. 2012). Future investigations can elucidate whether pteridines and other pigment molecules in the skin or circulating carotenoids in the blood may correlate with conspicuousness instead.

Many studies have delineated an endocrine basis for colorful ornamentation (reviewed in Hill and McGraw 2006). Though brighter males of this population appear to be more aggressive (Crothers et al. 2011; Crothers and Cummings, *in review*), we find here that brighter males did not have higher baseline circulating testosterone levels. Several mechanisms may explain the lack of relationship between these traits. Though testosterone can correlate with amphibian calling behavior (Marler and Ryan 1996; Emerson 2001) and induce changes in color pattern (Richards 1982; Hayes and Menendez 1999), the effects of testosterone on amphibian aggression have not been well investigated (Wilczynski et al. 2005). It is possible that differences in circulating testosterone levels between males may only be detected when the hormone is rapidly modulated during short-lived periods of social instability, as it is in other taxa (Wingfield et al. 1990; Goyman et al. 2007). However, we were unable to test the "challenge hypothesis" (Wingfield et al. 1990) with our dataset due to males' exceptionally minute blood volumes. Our experimental methodology may also have precluded us from observing subtle differences in testosterone between males because of stress-induced changes in their hormone profiles. Furthermore, though the bright warning signal in this species has evidently been co-opted as a sexual signal, the basic coloration and patterning of various *O. pumilio* morphs are present prior to sexual maturation in both males and females (L.R. Crothers, *pers. obs.*), and thus may not follow the physiological underpinnings common to many classic sexual signals. Finally, and contrary to common perception, the maintenance of conspicuous signals is often not testosterone-dependent (Owens and Short 1995) and other hormones have been implicated in the control of both conspicuous male signals and male behavior. Corticosterone (Moore and Jessop 2003; Cote et al. 2010) and melanocortins (Ducrest et al. 2008) both exhibit complex relationships with aggression and male signal expression in other taxa, but their impact on amphibian aggression and coloration are less understood (Wilczynski et al. 2005). Exogenous supplementation of arginine vasotocin has recently been shown to increase aggressive calling

in the neotropical frog *Eleutherodactylus coqui* (Ten Eyck and ul Haq 2012), so may be an especially promising candidate hormone for control of aggression in *O. pumilio*.

Poison frogs sequester their toxic alkaloids from the arthropods they eat (reviewed in Saporito et al. 2009, 2012), and we find here that there is a significant negative relationship between brightness and the level of chemical defense within males of this population. Brighter than average males had a lower diversity of alkaloids and exhibited a marginal trend for lower alkaloid quantities in their skin (Fig 4.4a). Brighter males also had less aggregate pumiliotoxin, which is considered the major class of toxic alkaloids in the genus (Fig 4.4b). Interestingly, we also detected correlations between the proportions of the most common alkaloids (pumiliotoxins and tricyclics) and the most common carotenoids (beta-carotene and xanthophylls) found within these males. There has been some speculation that the alkaloid sequestration and chemical modification performed by chemically defended species is oxidatively costly (Ahmad 1992; Blount et al. 2009), and the negative correlations we observe here between particular carotenoids and alkaloids (Fig 4.3b,c) provide indirect support for this hypothesis, even though total carotenoids and total alkaloids did not co-vary (Kendall's rank correlation: $N = 19$, $z = 0.105$, $P = 0.916$).

There has been much disagreement over whether warning signals should be quantitatively honest, with a relatively tight correlation between conspicuousness and toxicity, versus qualitatively honest, where the presence of a signal, regardless of its magnitude, sufficiently advertises secondary defense (Speed and Ruxton 2007; Blount et al. 2009; Speed et al. 2010; Lee et al. 2011). Theoretical investigations have predicted both negative and positive relationships between toxicity and conspicuousness (Speed and Ruxton 2007; Blount et al. 2009; Holen and Svennungsen 2012), and the few empirical studies performed investigating toxicity and conspicuousness have yielded seemingly conflicting results, both at the multi-species level (e.g., Summers and Clough 2001; Darst et al. 2006; Cortesi and Cheney 2010), and intraspecifically (Daly and Myers 1967; Bezzerides et al. 2007; Maan and Cummings 2012; reviewed in Speed et al. 2012). Furthermore, it appears that the relationship between toxicity and color can be relatively stochastic across time (as suggested by Daly et al. 2002; Saporito et al. 2006; Saporito et al. 2007). Here, we find that although there is a positive relationship between brightness and toxicity *across* phenotypically distinct

O. pumilio archipelago populations (Maan and Cummings 2012), there is evidently a negative relationship between these traits *within* the Solarte population. Our assessments of toxicity are based on alkaloid identification and quantification, and assume that having greater aggregate pumiliotoxin imparts greater noxiousness/toxicity to these frogs. However, though pumiliotoxins are commonly considered the important toxic class of alkaloids in this genus, it is not known which particular alkaloids may be most important in chemical defense.

Several phenomena may explain our finding that brighter males have less alkaloid diversity and aggregate pumiliotoxin in their skin, including brighter males being sampled by predators more frequently because they differ behaviorally or visually from duller males (Crothers et al. 2011; Crothers and Cummings 2013), a metabolic tradeoff between brightness and toxicity (Blount et al. 2009), or a correlation between brightness and age. Our past research (Crothers and Cummings 2013) suggests that many dull males of this population are cryptic at a distance to avian predators, and thus may require more chemical defense because they lack a conspicuous aposematic signal to deter predators. Furthermore, brighter males were caught at higher locations within their territories (Fig 4.2), providing some support for behavioral differences between bright and dull males. Finally, there may be a strategic trade-off between conspicuousness and toxicity, whereby individuals of a population can gain protection from predators through investing in either toxicity or conspicuousness, a pattern that has been observed across several poison frog species (Darst et al. 2006). Future studies can elucidate the relative importance of these explanations in driving the relationship between brightness and skin alkaloids within this population.

Our theoretical investigations with a model avian visual system revealed that birds cannot discriminate much of the brightness variation found within the Solarte population, implying that there may be little fitness tradeoff for brighter, less toxic males in terms of predation (Crothers and Cummings 2013). However, male warning signal brightness exhibits a negative relationship with dietarily based skin alkaloids, and thus may be a reliable indicator of foraging ability, territory quality, or perhaps genetic quality. Past findings that females prefer brighter males (Maan and Cummings 2009) are thus somewhat surprising in this context, since females may prefer a more conspicuous but physiologically compromised

male phenotype within this population. However, further investigations will need to be performed to see whether brighter males have higher mating success in this population.

A benefit of investigating signal evolution in *O. pumilio* is that it is evident that the processes of selection are all impacting a single, quantifiable trait: aposematic coloration. In the highly conspicuous and toxic Solarte population, warning signal brightness has seemingly been co-opted as a sexual indicator trait (Crothers and Cummings, *in review*). Many status-signaling models require that the signals be strategically costly in order to be evolutionarily stable (Berglund et al. 1996; Maynard Smith and Harper 2003). The sexually selected brightness within this population has previously been shown to correlate with body temperature and a call characteristic that contributes to mating success (Crothers et al. 2011), and there also appears to be a tradeoff between long-wave chroma (~redness) and brightness in some orange/red taxa, including this population (e.g., Grether 2000; Crothers and Cummings 2013). However, brightness does not appear to correlate with several classic measures of body condition, such as length-mass residuals (Crothers et al. 2011), circulating testosterone or pigments acquired from the diet. Instead, the costs may be imparted through a physiological measure unique to chemically defended species: alkaloids derived from the diet and sequestered in the skin. Future investigations may elucidate the physiological or strategic trade-offs driving the relationships we describe here.

4.5 TABLES

Carotenoid	Median (µg/g)	Mean (µg/g)	Range (µg/g)	S.D. (µg/g)	% Indiv.	Correlation Coefficient
Apocarotenoid	5.74	5.67	0-13.75	4.88	68	
Canary Xanthophyll	17.05	21.29	1.06-64.07	17.26	100	0.26
Canthaxanthin	6.05	11.93	0-73.74	16.73	79	0.22
Xanthophyll	3.77	6.47	0-24.78	6.73	84	0.48
cis-Ketocarotenoid	6.34	8.30	0-40.62	9.99	74	0.32
Echinenone	0	4.76	0-51.99	14.43	11	
3-Hydroxy-echinenone	34.41	47.31	0-179.22	43.29	95	0.27
Lutein Ester (1)	23.79	34.76	2.2-83.87	27.05	100	0.03
cis-Xanthophyll	18.84	22.26	0-71.22	20.03	84	0.12
Canary Xanthophyll Ester (1)	47.94	56.55	4.01-214.11	55.92	100	0.03
B-Carotene	152.63	195.08	37.96-482.83	134.50	100	0.11
Canary Xanthophyll Ester (2)	60.04	76.67	5.98-226.29	63.44	100	0.23
Ketocarotenoid Ester (2)	0	2.63	0-35.21	8.45	16	
Canary Xanthophyll Ester (3)	21.81	30.86	0-90.07	26.58	95	0.47
Canthaxanthin Ester	46.99	50.64	1.66-135.09	32.31	100	0.16
Ketocarotenoid Ester	18.82	24.77	0-95.38	22.81	84	0.25
Xanthophyll Ester	8.67	10.37	0-29.14	8.96	79	0.128
Total Carotenoids	459.73	610.32	86.04-1421.51	407.45	--	0.20

Table 4.1: Skin carotenoids found in Solarte males. The penultimate column presents the percentage of individuals in the dataset (N=19 males) that had the carotenoid present in their skin. The final column contains correlation coefficients for that carotenoid and male brightness (total reflectance flux) for those carotenoids found in 70% or more of males.

Alkaloid	Median (µg)	Mean (µg)	Range (µg)	S.D. (µg)	% Individuals	Correlation Coefficient
3,5-I 195B	0.00	0.087	0-0.877	0.216	20	
3,5-I 251K	0.00	0.010	0-0.202	0.045	5	
3,5-P 209K	0.00	0.002	0-0.049	0.011	5	
3,5-P 223H *(iso)*	0.00	0.479	0-8.509	1.896	25	
3,5-P 223H *(iso)*	0.00	0.009	0-0.166	0.037	10	
5,6,8-I 277E *(iso)*	0.00	0.009	0-0.172	0.038	5	
5,6,8-I 277E *(iso)*	0.00	0.001	0-0.022	0.005	5	
5,6,8-I 221U	0.00	0.001	0-0.019	0.004	5	
5,6,8-I 223A	0.00	0.036	0-0.439	0.104	20	
5,6,8-I 223X	0.00	0.001	0-0.015	0.003	5	
5,6,8-I 231B	0.00	0.004	0-0.024	0.007	25	
5,6,8-I 237C	0.00	0.109	0-1.424	0.320	40	
5,6,8-I 249C	0.00	0.002	0-0.034	0.008	5	
5,6,8-I 253H	0.00	0.021	0-0.235	0.056	25	
5,6,8-I 263A	0.00	0.007	0-0.122	0.027	10	
5,8-I *(unidentified)*	0.00	0.004	0-0.071	0.016	5	
5,8-I 195A	0.00	0.046	0-0.920	0.206	5	
5,8-I 195I	0.00	0.011	0-0.220	0.049	5	
5,8-I 205A	0.00	0.018	0-0.183	0.048	15	
5,8-I 207A	0.00	0.065	0-0.755	0.171	30	
5,8-I 221A	0.00	0.001	0-0.030	0.007	5	
5,8-I 223D	0.00	0.032	0-0.235	0.076	20	
5,8-I 233D *(iso)*	0.00	0.352	0-5.880	1.327	10	
5,8-I 233D *(iso)*	0.00	0.001	0-0.016	0.004	5	
5,8-I 233D *(iso)*	0.00	0.007	0-0.075	0.018	30	
5,8-I 233D *(iso)*	0.04	0.251	0-2.337	0.543	65	
5,8-I 233D *(iso)*	0.00	0.023	0-0.336	0.078	10	
5,8-I 235B	0.07	3.757	0-59.84	13.285	70	-0.03
5,8-I 243B	0.00	0.014	0-0.192	0.046	15	
5,8-I 245B	0.00	0.014	0-0.289	0.065	5	
5,8-I 251B *(iso)*	0.00	0.059	0-0.879	0.196	30	
5,8-I 251B *(iso)*	0.00	0.002	0-0.036	0.008	5	
5,8-I 253B	0.00	0.003	0-0.052	0.012	5	

Table 4.2: Skin alkaloids in Solarte males.

Isomers are indicated by the designation *(iso)*. The penultimate column contains the percentage of individuals in the dataset (N = 20 males) that had the alkaloid present in their skin. The final column contains the Kendall's tau rank correlation coefficient of that alkaloid and male brightness (total reflectance flux), for the few alkaloids found in 70% or more of males. (3,5-I: 3,5-disubstituted indolizidine; 3,5-P: 3,5-disubstituted pyrrolizidine; 5,6,8-I: 5,6,8-trisubstituted indolizidines; 5,8-I: 5,8-disubstituted indolizidines; aPTX: allopumiliotoxin; d-5,8-I: dehydro-5,8-disubstituted indolizidines; DHQ: 2,5 disubstituted decahydroquinolines; Lehm: lehmizidine; Pip: piperidine; PTX: pumiliotoxin; Spiro: spiropyrrolizidine; Tri: tricyclic).

Table 4.2 continued.

Alkaloid	Median (µg)	Mean (µg)	Range (µg)	SD (µg)	% Individuals	Correlation Coefficient
5,8-I 259B	0.00	0.003	0-0.067	0.015	5	
aPTX 305A *(iso)*	0.00	0.052	0-0.798	0.178	20	
aPTX 305A *(iso)*	0.00	0.001	0-0.027	0.006	5	
aPTX 323B	0.00	0.014	0-0.273	0.061	5	
aPTX 325A	0.00	0.291	0-4.892	1.095	25	
aPTX 325B	0.00	0.017	0-0.346	0.077	5	
d-5,8-I 221V	0.00	0.003	0-0.054	0.012	5	
d5,8-I 201A	0.00	0.003	0-0.039	0.010	10	
DHQ 195A *(iso)*	0.00	0.005	0-0.106	0.024	5	
DHQ 195A *(iso)*	0.10	2.852	0-37.37	8.314	75	-0.12
DHQ 195A *(iso)*	0.00	0.011	0-0.205	0.046	15	
DHQ 211A	0.00	0.123	0-1.352	0.317	25	
Izidine 209D	0.00	0.002	0-0.037	0.008	5	
Izidine 211C	0.00	0.002	0-0.015	0.005	20	
Lehm 275A	0.00	0.003	0-0.058	0.013	5	
Pip 211J	0.00	0.004	0-0.076	0.017	5	
PTX 251D	0.00	0.026	0-0.513	0.115	5	
PTX 277B	0.00	0.018	0-0.238	0.058	10	
PTX 307A	0.66	2.356	0-21.02	5.107	75	-0.33
PTX 307B	0.00	0.090	0-1.176	0.265	35	
PTX 307F *(iso)*	0.00	0.007	0-0.109	0.024	15	
PTX 307F *(iso)*	0.00	0.022	0-0.222	0.067	10	
PTX 307G	0.00	0.013	0-0.250	0.056	5	
PTX 309A	0.00	0.027	0-0.196	0.060	20	
PTX 321A	0.00	0.236	0-4.420	0.986	15	
PTX 323A *(iso)*	0.00	0.801	0-4.549	1.239	45	
PTX 323A *(iso)*	0.00	0.001	0-0.015	0.003	5	
PTX 323A *(iso)*	0.00	0.046	0-0.455	0.112	20	
PTX 323A *(iso)*	0.04	1.495	0-10.60	3.196	50	
PTX 323A *(iso)*	0.00	0.006	0-0.082	0.020	10	
PTX 325B	0.00	0.027	0-0.330	0.084	10	
Spiro 236	0.00	0.011	0-0.219	0.049	5	
Spiro 252A	0.00	0.019	0-0.258	0.057	30	
Tri ***(unidentified)***	0.00	0.003	0-0.061	0.014	5	
Tri 191B *(iso)*	0.00	0.005	0-0.029	0.010	25	
Tri 191B *(iso)*	0.57	0.555	0-1.214	0.297	90	0.04
Tri 203B *(iso)*	0.00	0.039	0-0.517	0.126	10	
Tri 203B *(iso)*	0.01	0.092	0-0.367	0.140	55	
Tri 205B *(iso)*	0.03	0.200	0-0.952	0.313	55	
Tri 205B *(iso)*	0.25	0.256	0.166-0.53	0.076	100	0.02
Tri 205H	0.00	0.001	0-0.019	0.004	5	
Unclassified 279I	0.00	0.072	0-0.812	0.197	20	
Unidentified	0.00	0.011	0-0.127	0.034	10	
Unidentified	0.00	0.004	0-0.084	0.019	5	

4.6 FIGURES

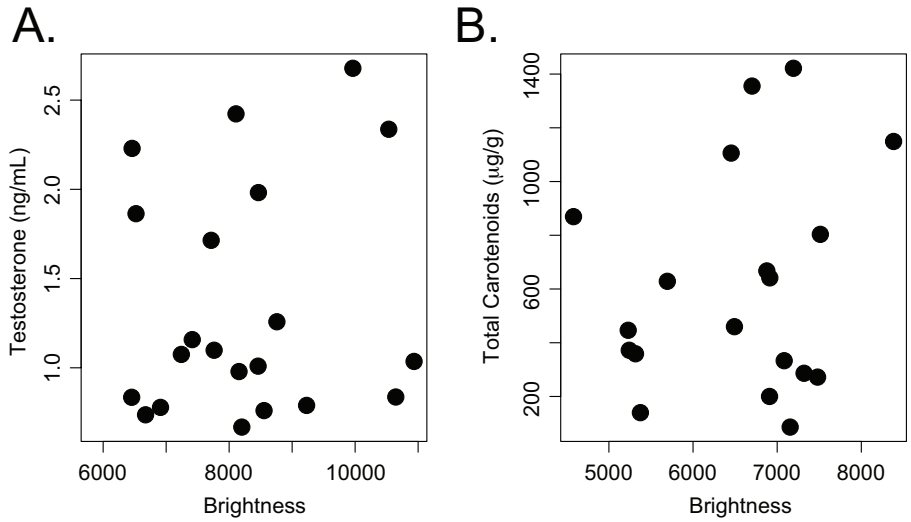

Figure 4.1: Relationships between male brightness (total reflectance flux) and (A) circulating testosterone and (B) total carotenoids in the dorsal skin.

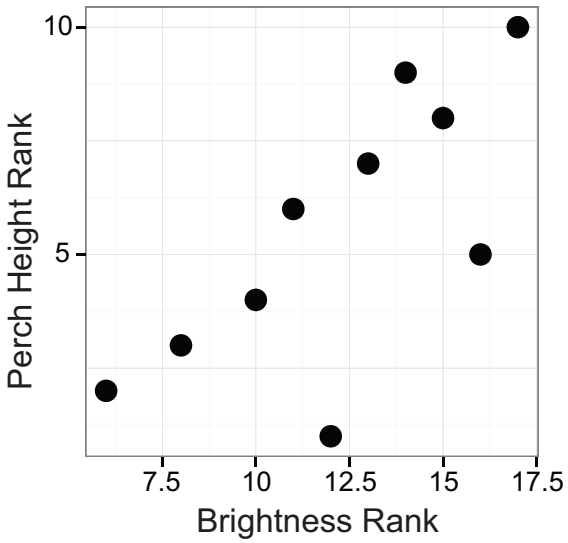

Figure 4.2: Ranks of male brightness and their perch height in their territories in the field at time of capture. Kendall's rank correlation: T = 36, P = 0.017. Larger rankings indicate larger values.

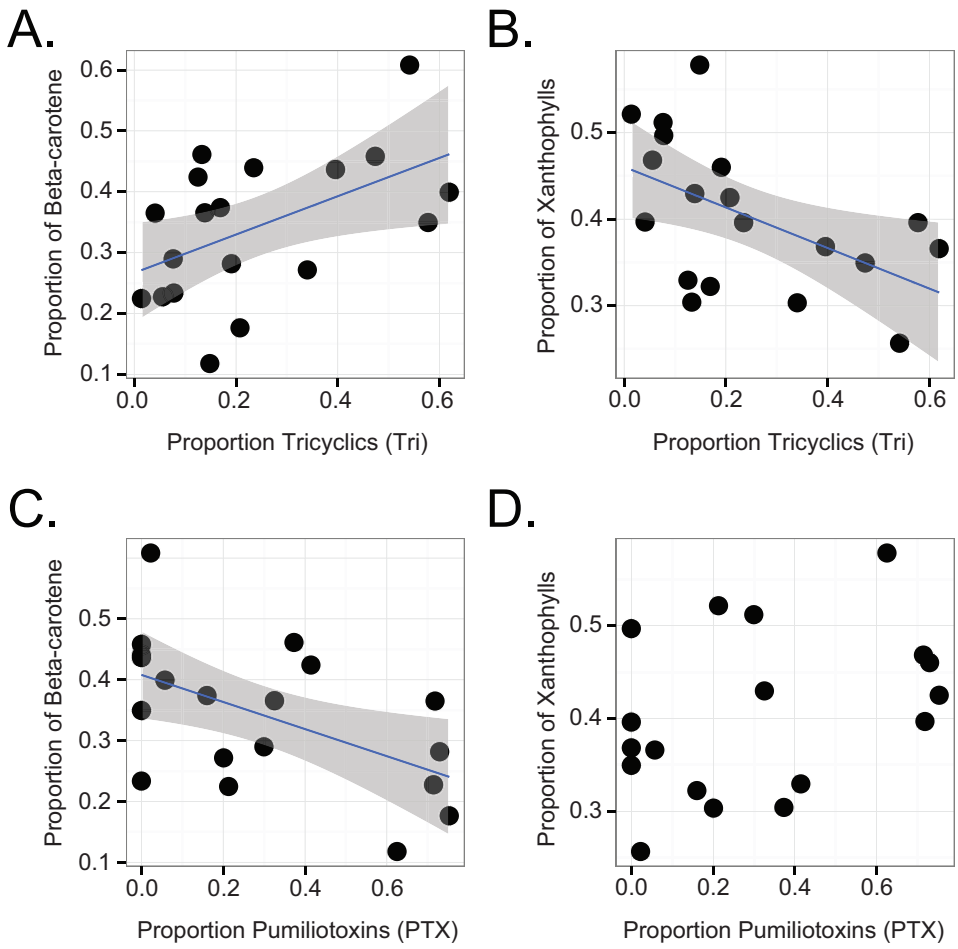

Figure 4.3: Relationships of the most common alkaloids and carotenoids found in Solarte male skin samples. Proportional tricyclic alkaloids (x-axis) and (A) beta-carotene and (B) xanthophylls (y-axes). Proportional pumiliotoxins (x-axis) and (C) beta-carotene and (D) xanthophylls. Line and shaded area flanking the line represent the predicted line and smoothed 95% confidence intervals of the linear model, respectively, while dots represent data points.

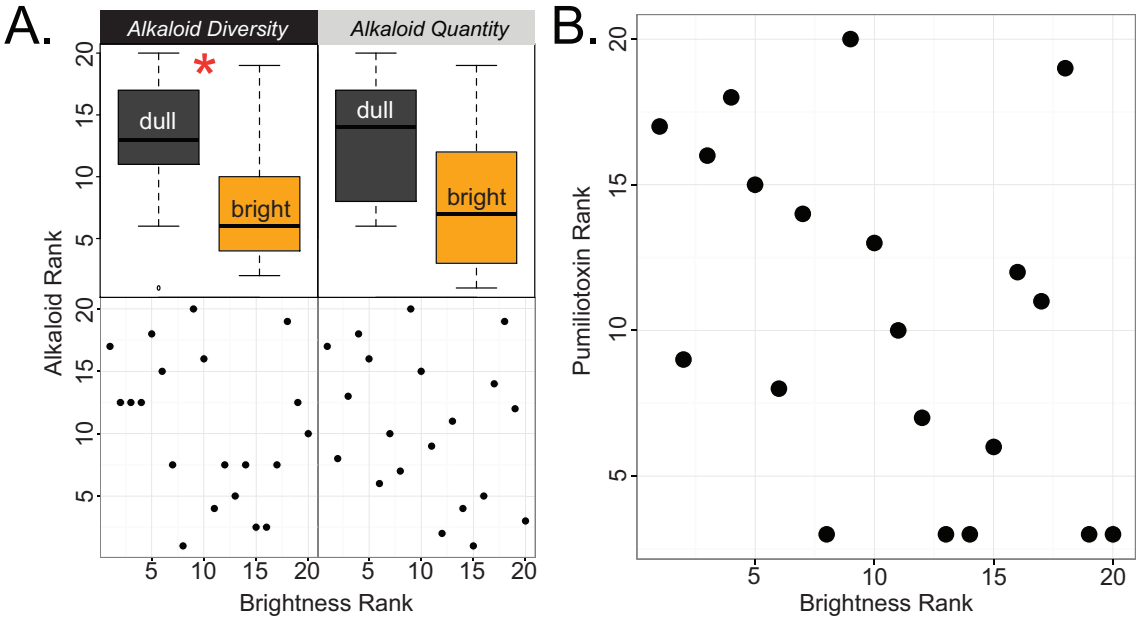

Figure 4.4: Relationships between male brightness and skin alkaloids. (A) Male brightness (***top***: as a dichotomous measure; ***bottom***: as a rank measure) and rankings of alkaloid diversity (***left***: the number of unique alkaloids present in the skin) and alkaloid quantity (***right***: total μg of alkaloids present in the standard-sized skin samples) in the dataset of 20 males. Larger rankings indicate larger values. (B) Significant negative relationship between ranked male brightness and ranked total pumiliotoxin (Kendall's rank correlation: N=20, z=-2.160, P=0.031).

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CONCLUSION

This research demonstrates that the selective agents impacting warning signal brightness within this species are at minimum three-fold. Natural selection by predators appears to impose a selective regime that maintains warning signal reliability across the phenotypically diverse and variably toxic populations of the archipelago (Maan and Cummings 2012; Cummings and Crothers 2013). There is also ample evidence that females are selecting on male coloration and brightness through both positive assortative mating and directional selection on brightness (Summers et al. 1999; Maan and Cummings 2008, 2009). Finally, my dissertation research provides strong evidence that the conspicuous warning signal of this species can be co-opted as a male status signal (**Chapters 2 and 3**). And surprisingly, the socially important brightness variation found within the Solarte population may be undetectable to the presumed major predator, birds, allowing intraspecific selection to work "under the radar" of some predators within these populations (**Chapter 1**). Thus, complex feedbacks between these three aspects of selection may have driven the impressive signal variation that rapidly arose as the Bocas del Toro archipelago formed over the course of the past ~9,000 years.

The research presented throughout this dissertation provides several significant contributions to the study of aposematic diversification in poison frogs, and to the study of signal evolution in general. First, this research presents evidence that conspicuous warning signals may be selected upon by both forms of conspecific "eavesdroppers" (conspecific competitors and potential mates). Therefore, the phenotypic diversity identified across a wide swath of aposematic taxa could be driven just as much by sexual selection as it is by patterns of natural selection (West-Eberhard 1983; Zamudio and Sinervo 2000; Seehausen and Schluter 2004; Dijkstra et al. 2005; Pryke and Griffith 2006). Second, we find that a conspicuous signal that has arisen through natural selection can evolve to function as an agonistic indicator trait, used by males to assess rivals before participating in costly fights.

Finally, in **Chapter 4** I present evidence that warning signal brightness exhibits physiological correlates different from those of classic sexual signals. While it does not appear to correlate with circulating testosterone or total skin carotenoids in the population

we studied, we did observe a negative relationship between male brightness and two aspects of chemical defense: alkaloid diversity and aggregate pumiliotoxins in the skin (considered the major toxic alkaloid in these frogs). Given our findings that brighter males are more aggressive and call from higher locations in their territories, they may be sampled more frequently by naïve predators. Our findings also provide some empirical support for a hypothesis by Blount et al. (2009) that signal brightness can function as a reliable indicator of defensive levels in aposematic organisms.

In Cummings and Crothers (2013), we argued that the diversification of signals in the archipelago relative to the common mainland morph could be due to two non-mutually exclusive scenarios: *(1)* predators impose a selective regime whereby populations above a toxicity-brightness threshold are at liberty to diversify via sexual selection and below which populations are constrained to maintain a stricter resemblance to a more cryptic population mean, and *(2)* synergistic/additive effects of inter- and intrasexual selection drive the evolution of brighter males within populations above the toxicity threshold. We predicted that predators exhibit purifying selection in populations that are weakly defended and exhibit greater tolerance of signal variation in populations that are strongly defended. We would expect that as particular populations lose their toxicity (perhaps due to differences in arthropod communities from the mainland populations; Saporito et al. 2006; Saporito et al. 2007) and develop more cryptic color patterns, individuals exhibiting aposematic signal deviations would be more vulnerable to detection by predators. Thus in less toxic, more cryptic populations, sexual selection for brightness should be constrained by purifying selection by predators. In populations that are strongly defended, however, predators may largely be tolerant to signal variation due to predator generalization (Darst and Cummings 2006) or perceptual limitations (e.g., **Chapter 1**). Hence, diversification via sexual selection in populations that are more toxic than the common mainland morph may be less subject to purifying selection and more likely to be tolerated by predators.

Our examination of the relative toxicity and conspicuousness of 10 different morphs in the Bocas del Toro archipelago, presented in Cummings and Crothers (2013), shows an agreement with the above predictions. Firstly, we found that the population representing the most common mainland phenotype (Almirante) exhibits trait values that are

closest to the mean for both toxicity and conspicuousness. Secondly, populations that deviate from the common mainland phenotype by exhibiting greater toxicity are also more conspicuousness to a bird predator's visual system, whereas those that have deviated from the common mainland phenotype towards being less toxic are also more cryptic. Finally, the relative degree of divergence from the common mainland phenotype is greater for the conspicuous morphs than the cryptic morphs, possibly due to the synergistic/additive effects of both natural and sexual selection (Cummings and Crothers 2013).

If natural selection is imposing a constraint on diversification of morphs in populations below some defense (toxicity) threshold, we may expect the role of sexual selection on aposematic signal evolution to be greatest in populations that exceed some critical defense threshold, such as the Solarte population studied throughout this dissertation. Sexual selection has been a potent force of signal divergence in other species (West-Eberhard 1983; Zamudio and Sinervo 2000; Seehausen and Schluter 2004; Dijkstra et al. 2005; Pryke and Griffith 2006), and there are several scenarios in which it could have been a major driver of *O. pumilio*'s phenotypic divergence, such as drift in the female preference trait or directional selection driven by female preference and/or or male-male interactions paired with interdependence between aposematic signal components (brightness and hue).

Directional sexual selection for brighter phenotypes by female preference or male-male interactions may have driven diversification of color patterns in the archipelago populations due to the interdependence of brightness and color (Maan and Cummings 2009). Brightness and hue (color) are interdependent features of a visual signal, and changes in signal brightness may lead to changes in signal hue. In general, creating a brighter visual signal can be achieved via two non-mutually exclusive design principles: increasing the peak reflectance (reflecting more light at a given wavelength), or increasing the spectral width of reflectance (reflecting light over a broader wavelength range). The increased brightness of the most exceptionally bright derived populations (Solarte and Bastimentos) exemplifies both principles: greater peak reflectances and shorter cut-on wavelengths (wavelength at which reflectance begins to exceed baseline), as compared with the common mainland morph (Almirante; Maan and Cummings 2009). The implication of this interdependence

between brightness and hue is that directional selection on one aspect of the aposematic trait (brightness) may cause a concomitant change in the other (hue), leading to the brighter colors observed on the islands of Bocas del Toro (orange and yellow). Many of the *O. pumilio* morphs inhabiting the islands of the Bocas del Toro region are brighter than the mainland morph, suggesting that sexual selection on brightness may have been a potent force in the signal evolution of populations of the archipelago (Maan and Cummings 2009).

The findings I present in this dissertation show that in the exceptionally conspicuous and toxic Solarte population, both males and females are assessing potential mates and rivals along the same trait axis (brightness). Thus, the effects of intra- and intersexual selection are likely to be additive or reinforcing in this population, resulting in stronger directional selection on this trait. The fact that Solarte exhibits the greatest degree of divergence from the mean morph detectability to an avian predator is consistent with this hypothesis (Cummings and Crothers 2013). Future research may delineate whether sexual selection is important in cryptic populations, and the extent to which brightness has been co-opted as an agonistic male signal throughout the archipelago.

Synopsis of Findings

In **Chapter 1**, we used receptor noise-limited visual models to assess whether model predators (birds, snakes, and crabs) and conspecifics can detect the extensive brightness variation found within the Solarte population. Our surprising findings were that while conspecifics and two predators (snakes and crabs) can likely detect brightness differences between males in the population, the presumed major predator (birds) cannot. Though this was a theoretical study, it brings up several interesting hypotheses and questions. First, we can predict that as long as sexually mediated selection has a greater "resolution" than that of predators, a shift in population brightness can occur, with some predators being largely permissive to sexually selected brightness changes within a population. Additionally, brightness and hue (color) are interdependent features of a visual signal, thus sexually selected shifts in brightness can be accompanied by concomitant changes in hue, potentially explaining why color diversification occurred so rapidly in this species (Maan and Cummings 2009; Crothers and Cummings 2013).

In **Chapters 2** and **3,** we tested Solarte males' responses to the brightness of potential rivals. By carefully manipulating the perceived brightness of size- and brightness-matched stimulus males, we were able to control for any possible behavioral differences between males of varying inherent brightness. We observed not only that brighter stimulus frogs attracted more attention from our focal males, but also that a focal male's own brightness correlated with his behavior. Brighter focal males approached rivals more readily and directed more of their behaviors to the brighter stimulus frog. To gain further insights into the role that male brightness plays in male territorial disputes, we tested the outcomes of fights in more naturalistic settings, by placing males in dyadic contests in a neutral fight arena immediately after capture. We found again that brighter males initiated interactions more readily by calling faster, and that the magnitude of the brightness asymmetry between males in a pair predicted the outcomes of the interactions. Fights that escalated (both males called) had smaller brightness asymmetries, and when asymmetries in brightness were large the brighter male was more likely to be the sole aggressor. Additionally, we found that male brightness was a more reliable indicator of a male's motivation to fight than were body mass or body length. We also observed males in their territories, noting whether they were visited by conspecifics and how they reacted to a simulated male intruder. We did not find evidence that brighter males were more likely to have a conspecific in their territory, despite predictions that they may more often be visited by females and by males (Maan and Cummings 2009; Crothers et al. 2011). However, we did observe that brighter males oriented to the sound of our acoustic playbacks faster than duller males did.

Finally, in **Chapter 4** we sought to delineate physiological correlates of male brightness. Our past investigations (described in **Chapter 2**) indicate that brighter males have lower body temperatures and exhibit a lower call pulse rate (which may be a positive predictor of fitness in this species; Pröhl 2003). Furthermore, a common explanation for extensive phenotypic variation in a sexually selected trait is that the trait is condition-dependent, tied to an aspect of an organism's health or genetic quality. Interestingly, we did not find a relationship between two classic measures of condition-dependence: circulating testosterone levels or dietarily derived color pigments in the skin (total carotenoids). However, we did find that brighter males had lower skin alkaloid diversity (when brightness

was coded as a dichotomous measure), and less aggregate pumiliotoxins in their skin. Thus, brightness may exhibit a negative relationship with toxicity *within* this population, despite the positive relationship between brightness and toxicity *across* the populations of the archipelago.

	Perceptual Abilities (Chapter 1)	**Dichotomous Choice (Chapter 2)**	**Dyadic Contests (Chapter 3)**	**Male Territorial Observations (Chapter 3)**	**Conditional Correlates (Chapter 2,4)**
Predation	Birds likely cannot discriminate male brightness variation; other predators can				Brighter males perch higher in territory, appear to be less toxic
Female Mate Choice	Females can discriminate male brightness variation (also found in *Maan & Cummings 2009)*	Females prefer to associate with brighter males (found by *Maan & Cummings 2009*)		No evidence that brighter males are more frequently visited by conspecifics (*also L.R. Crothers, unpublished dataset*)	Brighter males have lower pulse rate (predictive of mating success). No correlation b/w brightness and classic sexual signal conditional correlates
Males' Own Behavior	Males can discriminate male brightness variation (see also Chpt. 2).	Brighter males approach other males faster	Brighter males call faster	Brighter males orient faster to the call of an intruder male in their territory	
Male-Male Interaction Outcomes		Males preferentially approach and call to brighter males	Some evidence that male brightness functions as a male agonistic indicator trait	No evidence that brighter males are more frequently visited by males *(also L.R. Crothers, unpublished data)*	

Table 5.1: Summary of novel results in the context of past *O. pumilio* research. Blue text indicates findings by other researchers. Red text indicates findings that did not corroborate past research predictions.

Future Directions

Each chapter's results bring up interesting questions to be addressed in future studies. The findings in **Chapter 1** imply that avian predators cannot detect the brightness

variation observed within the Solarte population, potentially allowing sexual selection to work freely on this phenotypic trait. An assumption of studies of aposematic variation is that predators will exert stabilizing or purifying selection by oversampling rare aposematic phenotypes (Kapan 2001; Noonan and Comeault 2009; Borer et al. 2010; Chouteau and Angers 2011; Comeault and Noonan 2011). However, in populations that exceed a particular toxicity+conspicuousness threshold, predators may generalize and avoid phenotypes that bear some loose resemblance to the population average (Pavlov 1927; Darst and Cummings 2006; Exnerova et al. 2006), or may exhibit neophobic behaviors and avoid unfamiliar phenotypes altogether, regardless of their relative resemblance to a common aposematic phenotype (Marples et al. 1998). Thus, predators may not differentially prey on exceptionally bright or exceptionally dull phenotypes within the Solarte population, even if they are able to distinguish between these phenotypes. Conversely, behavioral differences between brighter and duller males may allow for predators to select on this phenotypic trait even if they cannot perceive the signal differences. Experiments on predator perception and behavior, including optomotor studies or choice paradigms using bright stimuli mimicking Solarte phenotypic variation, will be the ultimate test of whether predators can or cannot detect these brightness differences. Problematically, there remains a paucity of empirical data on the predators of poison frogs. Some have suggested that birds may not actually be the major predator of poison frogs (e.g., Santos and Cannatella 2011), although studies of poison frog predation have rarely been performed in the Bocas del Toro archipelago. The generalizability of our findings will thus depend upon which predators most commonly select on warning phenotypes in *O. pumilio* and other poison frog populations.

The combined findings in **Chapters 2** and **3** indicate that brighter males are more aggressive and that warning signal brightness has been co-opted as an agonistic signaling trait. If males use brightness as a reliable cue to avoid costly fights, we would expect that fights that involved highly aggressive behaviors such as charges would be more likely to occur when males were closely matched in their brightness levels. However, fights that reached this level of escalation were rare, and we did not observe a clear pattern for any of the body traits we investigated to influence this outcome. It is possible that the effect of brightness on these behaviors is weak, and could be detected with a larger sample size.

Future investigations can further test whether brightness is an honest signal of fighting ability by assessing whether males with artificially enhanced brightness suffer more frequent attacks (as implied by **Chapter 2**) and possible declines in measures of health for bearing a signal that is unrepresentative of their true fighting ability (Møller 1987). Finally, these results provide a hypothesis as to how the two components of sexual selection (intra- and intersexual) will interact to impact signal brightness. Since both males and females appear to be assessing potential mates and rivals along the same trait axis (brightness) that provides both sexes with important information, the effect of intra- and intersexual selection is likely to be additive or synergistic, resulting in stronger directional selection. Wild pedigree studies can provide information on the mating success of males of varying brightness levels, and estimates for the heritability (and hence evolvability) of this trait.

Two hypotheses resulting from laboratory behavioral tests were not borne out by our field observations. We predicted that brighter males would be more frequently visited by females (Maan and Cummings 2009), and have more frequent encounters with males (Crothers et al. 2011). However, brighter males were not more likely to have conspecifics in their territory in our observations presented in **Chapter 3**, or in more extensive observations of males in their territories not presented here (L.R. Crothers, *unpublished data*). Wild pedigrees are more effective investigations of male fitness than short-term observations of conspecific visitation rates, and genetic studies may be the best way to determine whether brighter males do indeed sire more offspring in this and other *O. pumilio* populations.

Finally, the findings in **Chapter 4** come with surprising insights into the physiological correlates of male brightness and color in this population. Carotenoids, commonly considered the important class of pigment molecules contributing to orange and red coloration, did not co-vary with this trait. This leaves future investigations to determine whether other pigments, such as pteridines, or perhaps structural coloration contribute to warning signal expression in *O. pumilio*. Furthermore, our finding that aggressive, bright Solarte males did not have higher baseline testosterone levels is only a first step in describing the hormonal profiles of these frogs. For example, it is possible that testosterone differences only arise during short-lived periods of social challenge, and our experiment did not have the resolution to detect this change. Finally, we provide tantalizing evidence for a negative

correlation between male brightness and chemical defense within this highly conspicuous population, which represents one of the few studies investigating the relationship between conspicuousness and toxicity in a single aposematic population. Finally, much can still be learned about the costs and consequences of sequestering, storing, and metabolizing dietary alkaloids, and the relationships that we detected between particular alkaloids and carotenoids could indicate that the plentiful carotenoids found in the skin of these frogs is present not for coloration but to buffer the costly process of alkaloid sequestration and storage.

***Sections of the conclusion have previously been published as Cummings & Crothers, 2013. Evolutionary Ecology 27:693-710.**

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VITA

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This dissertation was typed by the author.